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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterized by synovial inflammation and joint deformity. The pathogenesis of RA is mediated by several immune cells (such as monocytes, granulocytes, T and B lymphocytes) that infiltrate the synovial membrane and secrete a complex network of pro-inflammatory cytokines, chemokines and other inflammatory mediators, which perpetuate the inflammatory process associated with bone resorption and damage. RA remains an incurable, progressive and debiliting disease for the great majority of patients despite profound evolution of the therapeutics options over the last decades. Indeed, the new biotechnological treatments for RA are administered parenterally, have safety issues and constitute a significant economic burden to national health services. Thus, the development of therapeutic strategies able to control both synovial inflammation and bone erosion, with a high rate of disease remission, low incidence of side effects and low production costs is still an unmet medical need in RA.

Celastrol, a bioactive component of the Chinese herb *Tripterygium wilfordii*, has shown significant anti-inflammatory properties *in vitro* and *in vivo*, mainly attributed to the regulation of cytokine and chemokine production, the inhibition of cell invasion and proliferation, osteoclast modulation and suppression of bone resorption. Our group has previously characterized the efficacy and safety of celastrol in an animal model of arthritis, showing a reduction of synovial leukocyte infiltration.

The main goal of this work was to analyze the effect of celastrol with or without LPS stimulation on primary human leukocytes (such as monocytes, granulocytes, T and B cells and their subsets) activation, maturation, survival and apoptosis using peripheral blood samples collected from chronic RA patients (with more than 1 year of disease duration) in comparison with healthy controls, using Flow Cytometry and performing alarmarBlue® cell vitality assay.

For that, we first proceeded to the optimization of the experimental conditions (assessing the optimal compounds concentrations and incubation time to be tested), where peripheral leukocytes from healthy controls (n=4) were incubated at a range of celastrol (0,001 µM, 0,01 µM, 0,05 µM, 0,1 µM, 0,3 µM, 0,5 µM and 1 µM) and LPS (2 µg/mL, 5 µg/mL and 10 µg/mL) concentrations, and under a varying incubation times (0h, 4h, 16h and 24h). After incubation, cell viability was assessed using alarmarBlue® by spectrophotometry assay, and an immunophenotyping characterization of monocytes,

granulocytes, B and T cells was performed by Flow Cytometry. We found that incubation for 4h with celastrol 0,3 μM and LPS 10 μg/mL was the condition that showed less lymphocyte's, monocyte's and granulocyte's cell death, appearing to be the best condition to be tested in the following experiments. Next, we studied the effect of celastrol in peripheral leukocytes of the recruited chronic RA patients (*n*=5) and healthy controls (*n*=10), using the pre-optimized experimental conditions.

Our results have shown that celastrol had no significant effect on CD14+ monocytes, CD66b+ granulocytes, CD19+ B cell and CD3+ T cell levels, both in healthy controls and in chronic RA patients.

Accordingly, the overall data about monocytes have shown that celastrol was able to diminish (after LPS stimulation) CD16 and CD115 frequency and expression, and restore HLA-DR expression and CD86 frequency and expression to basal levels in monocytes (on total CD14+ cells) from healthy controls. In addition, in chronic RA patients, celastrol was able to reduce CD115 frequency and decrease (after LPS stimulation) CD86 frequency on monocytes (on total CD14+ cells).

Regarding granulocytes, our results have shown that celastrol was able to diminish CD62L expression and restore CD11b expression and CXCR2 frequency to basal levels in granulocytes (on total CD66b+ cells) from healthy controls. In chronic RA patients, celastrol was able to restore CD11b expression to basal levels in granulocytes (on total CD66b+ cells).

Our data regarding T cells have shown that celastrol was able to increase central memory Th2-like cells (CXCR3-CCR6-) and effector memory Th2-like cells (CXCR3-CCR6-) frequency and restore naïve activated T helper cells (CD45RO-HLA-DR+) frequency to basal levels from healthy controls. In chronic RA patients, celastrol was able to increase memory activated T helper cells (CD45RO+HLA-DR+) frequency. Moreover, our results have shown that celastrol was able to reduce (after LPS stimulation) CXCR3 expression to basal levels in T cells (on total CD3+ cells and on T helper cells (CD3+CD4+)) from healthy controls.

Concerning B cells, our findings have shown that celastrol was able to diminish RANKL and CD95 frequency on transitional B cells (IgD+CD38++) and CD95 expression on naïve B cells (IgD+CD27-) and transitional B cells (IgD+CD38++) from healthy controls. In addition, celastrol was able to restore RANKL frequency to basal levels in B cells (on total CD19+ B cells, naïve (IgD+CD27-), pre-switch memory (IgD+CD27+), post-switch memory (IgD-CD27+) B cells)) from healthy controls. Also, in healthy controls,

celastrol was able to restore CD95 frequency and expression and FcgRIIB expression to basal levels in B cells (on plasmablasts (IgD-CD38++); restore CD95 frequency and CD21 expression to basal levels on naïve B cells (IgD+CD27-) and CD21 expression to basal levels in B cells (on total CD19+ B cells); and restore (after LPS stimulation) HLA-DR frequency to basal levels in B cells (on transitional B cells (IgD+CD38++).

Celastrol seems to have a stronger effect on innate immune system cells, reducing their overall activation, differentiation and migration potential.

Accordingly, celastrol is a promising candidate for further testing in the clinic for RA therapy. Furthermore, the results suggest that celastrol might also be beneficial for the treatment of a few other autoimmune diseases besides arthritis.

Keywords: Rheumatoid arthritis; celastrol; leukocytes; inflammation; bone damage.

RESUMO

A artrite reumatóide (AR) é uma doença progressiva autoimune caracterizada por inflamação crónica sinovial e deformação irreversível das articulações, que pode levar à destruição da cartilagem e erosão óssea, causando incapacidade funcional e redução da esperança média de vida do doente, se não for devidamente e precocemente tratada. Inicialmente, manifesta-se através de inchaço, dor e rigidez das pequenas articulações das mãos, alastrando-se progressivamente para as articulações maiores dos joelhos e tornozelos. Deste modo, o diagnóstico e tratamento precoce e adequado é essencial para prevenir a progressão da doença e a destruição óssea e articular a ela associada.

O processo inflamatório da AR é caracterizado pelo recrutamento de células imunitárias (leucócitos) da circulação sanguínea periférica para o líquido e tecido sinovial. Nesta patologia estão envolvidas células imunitárias tanto do sistema imunitário inato (macrófagos e neutrófilos) como do adaptativo (linfócitos B e T), que infiltram a membrana sinovial (que reveste as articulações) e secretam citocinas pro-inflamatórias – particularmente as interleucinas (IL)-1β, IL-6 e IL-17 e o factor de necrose tumoral (TNF) – , quimiocinas e outros mediadores inflamatórios, tornando-a hiperplásica. Assim, o tecido sinovial torna-se, maioritariamente, repleto de macrófagos e linfócitos (células B e T), enquanto que o líquido sinovial é concentrado com neutrófilos.

As interações entre células apresentadoras de antigénios (APCs), células T e células B activam a resposta imunitária, levando à produção de autoanticorpos e excessiva activação de células T. Subsequentemente, estas células T activadas vão continuamente activar monócitos, macrófagos e fibroblastos sinoviais, induzindo a produção de citocinas pro-inflamatórias e quimiocinas que perpetuam o processo inflamatório, bem como mediadores de actividade óssea e enzimas com capacidade de reabsorver e destruir cartilagem e osso. Monócitos/macrófagos (precursores de osteoclastos) são induzidos a expressar RANK, que após se ligar ao seu ligando, RANKL, secretado pelos osteoblastos, estimula a sua diferenciação em osteoclastos maduros, responsáveis pela reabsorção e erosão óssea na AR.

A AR é uma doença de etiologia desconhecida e que afecta cerca de 0,5% a 1% da população mundial, sendo mais frequente nas mulheres (≈75%). Apesar da profunda evolução das opções terapêuticas ao longo das últimas décadas, a AR continua a ser uma doença incurável, progressiva e debilitante para a grande maioria dos doentes. O objectivo do tratamento na AR é a remissão, que é alcançada apenas em 20% - 30% dos doentes, se

precocemente e adequadamente tratados, no entanto, a maioria experiencia efeitos colaterais graves. Os primeiros tratamentos administrados aos doentes com AR são glucocorticóides, úteis no alívio dos sintomas clínicos e na diminuição da inflamação. Contudo, por terem efeitos secundários adversos em doses elevadas, são substituídos, numa segunda fase por drogas antirreumáticas modificadoras da doença (csDMARDs) sintécticas convencionais, que incluem o metrotexato (MTX), o fármaco mais usado no tratamento da AR e eficaz no controlo da actividade e progressão da doença. Ainda assim, uma grande percentagem de doentes é não-respondedora ou deixa de responder ao tratamento, pelo que a introdução de terapias biológicas (bDMARDs), como agentes anti- TNF, IL-1β, IL6, células B e T, têem surgido como uma alternativa aos convencionais DMARDs. Mais recentemente, têem também sido desenvolvidos agentes inibidores que são pequenas moléculas (tsDMARDs), como o tofacitinib, que apesar de inovadoras, infelizmente são terapias excessivamente caras, limitando o acesso aos serviços de saúde. Para além disso, muitas destas terapias são apenas eficazes no controlo da inflamação ou no do dano ósseo, pelo que para alguns doentes nenhuma destas terapias é eficaz. Assim, o desenvolvimento de estratégias terapêuticas capazes de controlar tanto a inflamação sinovial como a erosão óssea, com uma elevada taxa de remissão da doença, baixa incidência de efeitos colaterais e baixos custos de produção ainda é uma necessidade médica não atendida na AR.

O celastrol, um componente bioactivo extraído da planta chinesa *Tripterygium wilfordii*, que tem sido muito usado na medicina Chinesa para tratar inflamação e doenças auto-imunes incluindo a AR, foi previamente identificado pelo nosso grupo como um potencial candidato terapêutico para a AR, por inibir simultaneamente a produção das citocinas pro-inflamatórias TNF e IL-1β, directamente relacionadas com a inflamação e a degradação óssea na AR. Vários estudos têem apontado significantes propriedades anti-inflamatórias do celastrol *in vitro* e *in vivo*, maioritariamente atribuídas à regulação da produção de citocinas e quimiocinas, à inibição da invasão/infiltração e proliferação celular, modulação de osteoclastos e supressão da reabsorção óssea. Particularmente, o nosso grupo tem determinado a eficácia e segurança do celastrol num modelo animal de artrite, mostrando uma redução da infiltração leucocitária sinovial.

Assim, o principal objectivo deste trabalho foi avaliar o efeito do celastrol, com ou sem estimulação de LPS, na activação, maturação, sobrevivência e apoptose de leucócitos humanos primários (monócitos, granulócitos, células B e T e suas subpopulações), utilizando amostras de sangue periférico recolhidas de doentes com AR crónica (com mais de 1 ano de duração de doença) em comparação com controlos saudáveis, recorrendo à técnica de Citometria de Fluxo

e ao ensaio de viabilidade alamarBlue®.

Para isso, primeiramente procedemos à optimização das condições experimentais (com vista à determinação das concentrações ideais de compostos e tempo de incubação a testar), onde leucócitos periféricos de controlos saudáveis (*n*=4) foram incubados numa gama de concentrações de celastrol (0,001 µM, 0,01 µM, 0,05 µM, 0,1 µM, 0,3 µM, 0,5 µM e 1 µM) e LPS (2 µg/mL, 5 µg/mL e 10 µg/mL), e sob tempos de incubação variáveis (0h, 4h, 16h e 24h). Após cada incubação, a viabilidade celular foi determinada usando o reagente de alamarBlue® através de um ensaio de espectrofotometria, e a caracterização imunofenotípica dos monócitos, granulócitos, células B e T, foi realizada por Citometria de Fluxo. De entre as condições testadas, verificou-se que a incubação de 4h com 0,3 µM de celastrol e 10 µg/mL de LPS foi a condição que apresentou menos morte celular das populações de linfócitos, monócitos e granulócitos, surgindo como a condição ideal a ser testada nas experiências seguintes. Em seguida, procedemos ao recrutamento de doentes com AR crónica (*n*=5) e controlos saudáveis (*n*=10) e estudámos o efeito do celastrol em leucócitos periféricos, testando as condições experimentais pré-optimizadas.

O nosso trabalho demonstrou que o celastrol não teve efeito significativo nos níveis de células CD14+ (monócitos), CD66b+ (granulócitos), CD19+ (células B) e CD3+ (células T), tanto nos indíviduos saudáveis como nos doentes com AR crónica.

Em relação aos monócitos, o celastrol mostrou ser capaz de diminuir (após estimulação com LPS) a frequência e a expressão de CD16 e CD115, e restaurar a expressão de HLA-DR e a frequência e expressão de CD86 para níveis basais nas células CD14+ totais de controlos saudáveis. Além disso, nos doentes com AR crónica, celastrol foi capaz de reduzir a frequência de CD115 e diminuir (após estimulação com LPS) a frequência de CD86 nas células CD14+ totais.

No que diz respeito aos granulócitos, o celastrol foi capaz de reduzir a expressão de CD62L e restaurar a expressão de CD11b e a frequência de CXCR2 para níveis basais nas células CD66b+ totais de controlos saudáveis. Nos doentes com AR crónica, o celastrol foi capaz de restaurar a expressão de CD11b para níveis basais nas células CD66b+ totais.

Quanto às células T, o celastrol foi capaz de aumentar a frequência de células central memory Th2-like (CXCR3-CCR6-) e effector memory Th2-like (CXCR3-CCR6-) e restaurar a frequência das naïve activated T helper (CD45RO-HLA-DR+) para níveis basais de controlos saudáveis. Nos doentes com AR crónica, o celastrol foi capaz de aumentar a frequência de células memory activated T helper (CD45RO+HLA-DR+). Para além disso, o celastrol foi capaz de reduzir (após estimulação com LPS) a expressão de CXCR3 para

níveis basais nas células CD3+ totais e nas T helper (CD3+CD4+) de controlos saudáveis.

No que concerne às células B, o celastrol foi capaz de diminuir a frequência de RANKL e CD95 nas células transitional (IgD+CD38++) e a expressão de CD95 nas naïve (IgD+CD27-) e transitional (IgD+CD38++) de controlos saudáveis. Além disso, o celastrol foi capaz de restaurar a frequência de RANKL para níveis basais nas células CD19+ totais, nas naïve (IgD+CD27-), pre-switch memory (IgD+CD27+) e post-switch memory (IgD-CD27+) de controlos saudáveis. Nos doentes com AR crónica, o celastrol foi capaz de restaurar a frequência e expressão de CD95 e a expressão de FcgRIIB para níveis basais nos plasmablasts (IgD-CD38++); restaurar a frequência de CD95 e a expressão de CD21 para níveis basais nas células naïve (IgD+CD27-) e a expressão de CD21 para níveis basais nas células CD19+ totais; e restaurar (após estimulação com LPS) a frequência de HLA-DR para níveis basais nas células transitional (IgD+CD38++).

Celastrol parece ter um efeito mais forte sobre as células do sistema imune inato, reduzindo a sua activação geral, diferenciação e potencial de migração. Desta forma, celastrol é um candidato promissor para futuros ensaios clínicos em doentes com AR. Além disso, estes resultados sugerem que o celastrol também pode ser benéfico para o tratamento de algumas outras doenças autoimunes além da artrite.

Palavras-chave: Artrite reumatóide; celastrol; leucócitos; inflamação; dano ósseo.

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Olá! Tudo bem? ☺

ABBREVIATIONS

7-AAD	7-Amino-actinomycin D
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AIA	Adjuvant-induced arthritis
APCs	Antigen-presenting cells
BAFF-R	B cell activating factor -receptor
bDMARDs	Biologic disease-modifying anti-rheumatic drugs
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
c-Fms	Colony-stimulating factor-1 receptor
CIA	Collagen-induced arthritis
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
csDMARDs	Conventional synthetic DMARDs
CXCR	Chemokine (C-X-C motif) receptor
DAS28	Disease activity score of 28 joints
DFZ	Deflazacort
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FasR	Fas receptor
FBS	Fetal bovine serum
FcgRIIB	Fc gamma-receptor IIB
FVD	Fixable Viability Dye
FLSs	Fibroblast-like synoviocytes
Foxp3	Forkhead box protein 3
GCs	Glucocorticoids
HC	Healthy control
HIF-1α	Hypoxia-inducible factor-1α
HLA	Human leukocyte antigen
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage-colony stimulating factor
MFI	Mean fluorescence intensity

MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MTX	Methotrexate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activate B cells
NSAIDs	Nonsteroidal anti-inflammatory drugs
PDN	Prednisone
PBS	Phosphate Buffered Saline
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor-κB
RANKL	RANK ligand
RANTES	Regulated upon activation, normally T cell expressed and secreted
RBC	Red Blood Cell
RF	Rheumatoid factor
RORgT	Retinoic acid receptor-related orphan receptor gamma T
ROS	Reactive oxygen species
RT	Room temperature
SLZ	Salazopyrin
TCRs	T-cell receptors
Tc	T cytotoxic cells
Th	T helper cells
Th1	T helper 1 cells or IL-1-producing T cells
Th2	T helper 2 cells or IL-2-producing T cells
Th17	T helper 17 cells or IL-17-producing T cells
TNF	Tumor necrosis factor
tsDMARDs	Targeted synthetic disease-modifying anti-rheumatic drugs
Treg	Regulatory T cells
VAS	Visual Analogue Scale
WBCs	White blood cells

TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

CHAPTER I – INTRODUCTION

1. RHEUMATOID ARTHRITIS (RA)

1.1. DEFINITION

Rheumatoid arthritis (RA) is a progressive immune-mediated inflammatory disease characterized by chronic synovial inflammation, bone erosion and cartilage destruction in the joints. If not properly treated, RA leads to deformities, functional disability and reduced life expectancy (Wolfe FE and Hawley DJ, 1998; Pincus TE et al., 1994).

The most common symptoms of RA include symmetrical inflammation of small joints (hands and feet) and latter of larger joints (shoulders, elbows, hips, knees and ankles), accompanied by swelling, pain, morning stiffness and fatigue. This disease is often associated with other comorbidities, such as cardiovascular, pulmonary, psychological and skeletal disorders (Kourilovitch M et al., 2014; Michaud K et al., 2007).

The RA incidence is higher in individuals between 30 to 50 years of age, affecting more frequently women (≈75%), and is a relatively frequent disease with an overall world prevalence of 0,5% - 1% (Alamanos Y et al., 2005). In Portugal, RA affects about 0,7% of the population (Branco JC et al., 2016), which represents a significant impact on health systems (Laires PA et al., 2016).

Currently, RA diagnosis is performed according to the 2010 American College of Rheumatology/European League Against Rheumatism (2010 ACR/EULAR) criteria which focus on some patient parameters including: the clinical history of the patient; the joint involvement (the number of swollen or tender joint); the presence of autoantibodies in the serum, such as rheumatoid factor (RF) and/or anti-citrullinated protein antibody (ACPA); the raised levels of inflammatory markers in the blood such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR); the radiologic analyses and symptoms duration (Aletaha D et al., 2010). It is important to diagnose RA in the earliest possible phase of the disease course, since a prompt diagnosis and an accurate early therapeutic strategy are essential to prevent disease progression and joint erosions (Emery P et al., 1997).

1.2. ETIOLOGY

RA is a disease of unclear etiology, however, various factors including genetic and environmental factors are thought to promote RA development (Davidson A et al., 2001).

RA develops in a genetically susceptible host and some of the alleles associated with RA have been described. Human Leukocyte Antigen (HLA) emerge as the most important genetic factor for the susceptibility of this disease (Huizinga TW et al., 2005). HLA gene complex encodes the major histocompatibility complex (MHC) proteins, which are cell-surface proteins responsible for the immune regulation, by presenting foreign molecules (antigens) to T cells, leading to T cell activation.

HLA-DRB1 risk alleles are the most significant genetic susceptibility locus in RA, and genetic studies have shown that RA in the Caucasian population is strongly linked to the HLA-DRB1*01, HLA-DRB1*04 and HLA-DRB1*10 alleles (Raychaudhuri S et al., 2012). Moreover, regarding the environmental risk factors, several studies have reported that smoking is the most important risk factor for RA in individuals with HLA-DRB1 susceptibility alleles (Symmons DP et al., 1997), promoting the development of autoantibodies, RFs or ACPAs (Morgan AW et al., 2009; Klareskog L et al., 2006), which are predictor factors for a severe form of RA. The female gender, older age, lifestyle, diet, obesity and infectious agents amongst others, are also associated with an increased risk for developing RA (Gerlag DM et al., 2016; Alamanos Y et al., 2005).

1.3. PATHOPHYSIOLOGY

RA is characterized by leukocyte recruitment from the peripheral blood circulation into the synovial fluid and synovial tissue. This pathology involves a complex network of immune cells: macrophages and neutrophils from the innate immune system, and lymphocytes (T and B cells) from the adaptive immune system. These cells infiltrate the synovial membrane and secrete pro-inflammatory cytokines, particularly interleukins (IL)-1β, IL-6 and IL-17 and tumor necrosis factor (TNF), as well as the excess of other inflammatory mediators, including chemokines and soluble adhesion molecules (Mclnnes IB et al., 2007). Before these events take place, neutrophils migrate to the synovial fluid, where they phagocyte immune complexes and release powerful proteases (Nathan C, 2006). The RA synovial tissue is highly infiltrated with macrophages and lymphocytes (T and B cells) while the synovial fluid has large numbers of neutrophils (Harris ED., 1994) (**Figure 1**).

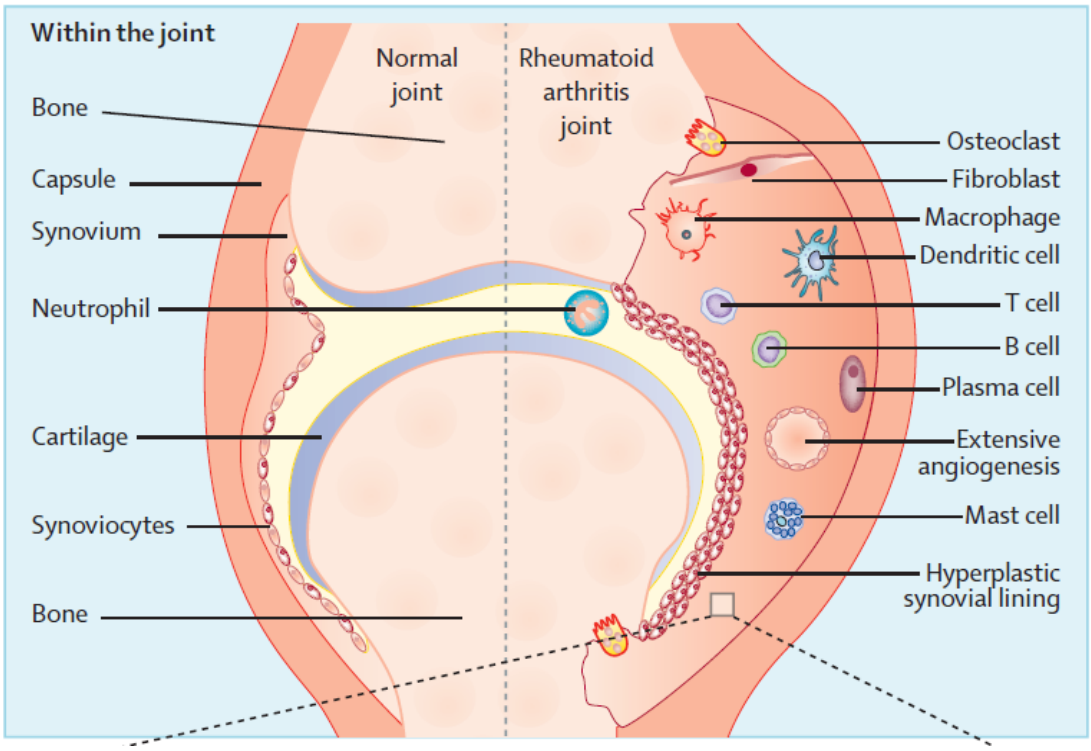

Figure 1 – Representation illustrating and comparing healthy (***Normal joint***) and arthritic (***RA joint***) articular joint. The ***RA joint*** shows increased inflammation and cellular activity (Smolen JS et al., 2016).

Aside from the synovial inflammatory cellular infiltration, the synovial tissue lining, composed of a thin layer of synoviocytes (macrophage-like and fibroblast-like synoviocytes (FLSs)), undergoes hyperplasic and expands, leading to a persistent inflammation associated with articular cartilage (Muller-Ladner U et al., 1996) and bone damage (Tolboom TCA et al., 2005).

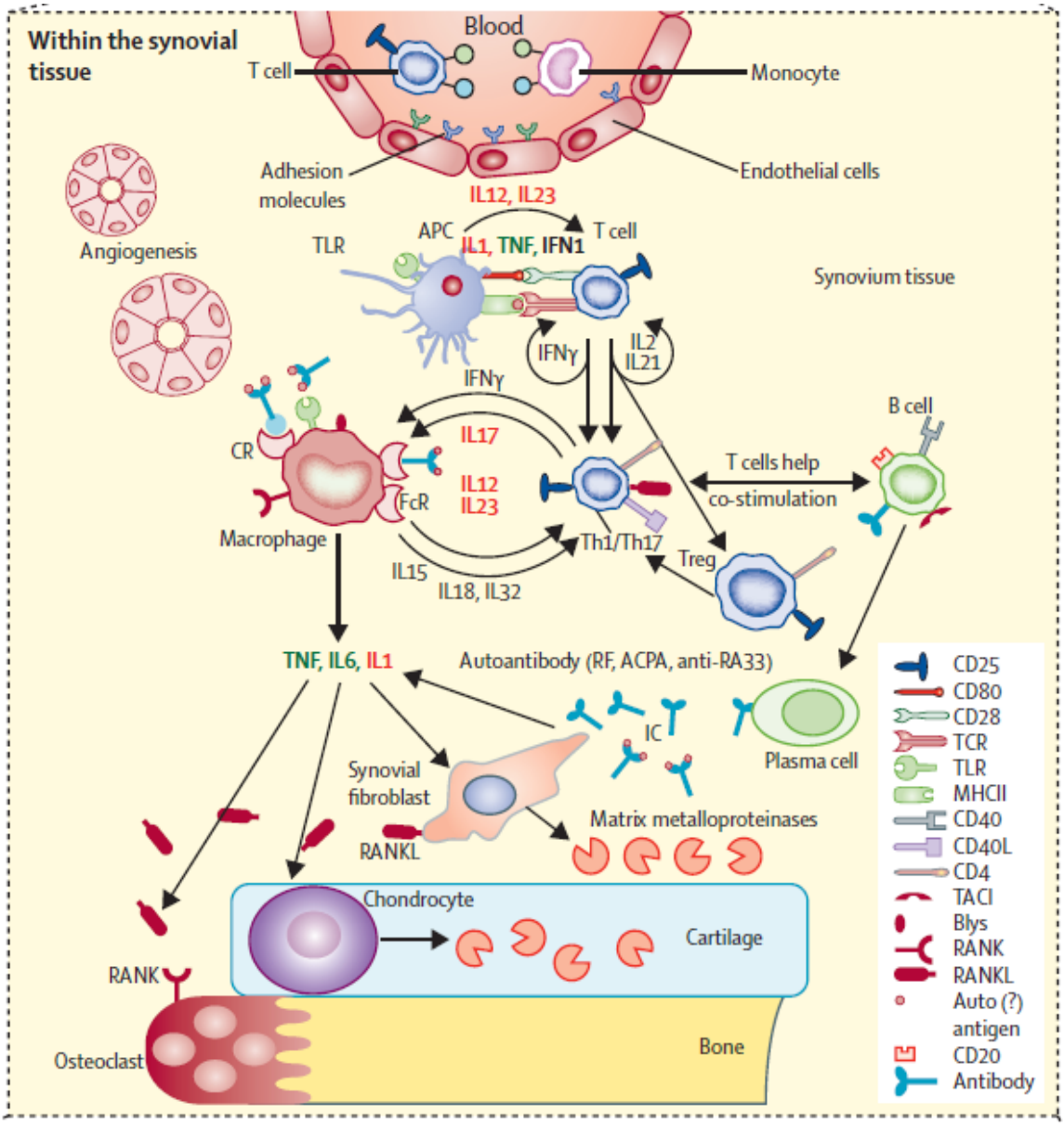

Figure 2 – Schematic representation of the inflammatory microenvironment in inflamed joints and events occurring in Rheumatoid Arthritis. The interactions among antigen-presenting cells (APCs), T cells and B cells would activate immune response, leading to the production of autoantibodies and excessive T cell activation. Subsequently, these T cells would continuously activate monocytes, macrophages and synovial fibroblasts, with up-regulated inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs). Macrophages might differentiate into osteoclasts, which would resorb and destroy bone. Blys – B lymphocyte stimulator. C' – complement. CP – citrullinated peptide. CR – complement receptor. FcR – receptor for the Fc portion of IgG. IC – immune complex. IFN – interferon. IFN1 – type 1 interferons. IL – interleukin. RF – rheumatoid factor. TACI – transmembrane activator and calcium-modulator and cyclophilin ligand interactor. TCR – T-cell receptor. Th1 – T-helper 1 cell. TLR – Toll-like receptor. Treg – regulatory T cell (Smolen JS et al., 2016).

The synovial hyperplasia phenomenon is named of the synovitis and is not only caused by the homing of these cells into the synovia, but also by the perpetuation of the inflammatory process due to the production of cytokines that maintain inflammatory cells activated (Harris ED et al., 1990) (**Figure 2**).

RA was classically considered mainly as a T-cell driven disease. Invading T cells are activated when it occurs the T-cell receptors (TCRs) interaction and signaling via costimulatory molecules, including Cluster of Differentiation (CD) 28 (on the T cells) binding to CD80/CD86 (on the APCs), which are essential for the initial T cell activation, leading to an upregulation of CD40L (Frauwirth KA et al., 2002; Janeway CAJ et al., 1994; Lenschow DJ et al., 1996). Then, T cells activated will lead to the activation of synovial monocytes, macrophages and synovial fibroblasts, through the production of interferon-gamma (IFN-γ) (Lundy SK et al., 2007; Kinne RW et al., 2007). CD40L interaction with CD40 on the APCs, also leads to activation of synovial monocytes/macrophages, fibroblast-like synoviocytes (FLS), and B cells, and may play a critical role in repeated activation of memory T cells in the synovium and, thus, maintenance of inflammatory reactions (Howland KC et al., 2000).

The subsequent overproduction of IL-1β (Ruscitti P et al., 2015; Wei S et al., 2005; Jules J et al., 2012), IL-6 (Axmann R et al., 2009; Yoshida Y et al., 2014), IL-17 (Lubberts E et al., 2005; Jovanovic DV et al., 1998; Yago T et al., 2009) and TNF (Azuma Y et al., 2000; Kanazawa K et al., 2005; Zhang YH et al., 2001) will induce monocytes/macrophages (osteoclast precursors) to express the receptor activator of nuclear factor (NF)-kB (RANK), which after binding to its ligand (RANKL) secreted by osteoblasts, stimulate their differentiation into mature osteoclasts that are responsible for bone resorption (**Figure 3**) (Pettit AR et al., 2001; Redlich K et al., 2002; Boyce BF et al., 2008). Differentiation of osteoclasts from their mononuclear precursors also requires macrophage-colony stimulating factor (M-CSF), engaging its receptor CD115 (c-Fms) on the surface of monocyte/macrophage precursors. Both RANKL and M-CSF are abundant in the synovium, providing the prerequisite milieu for osteoclastogenesis (osteoclast formation and maturation process) (Firestein G et al., 1988; Gravallese EM et al., 2000; Shigeyama Y et al., 2000).

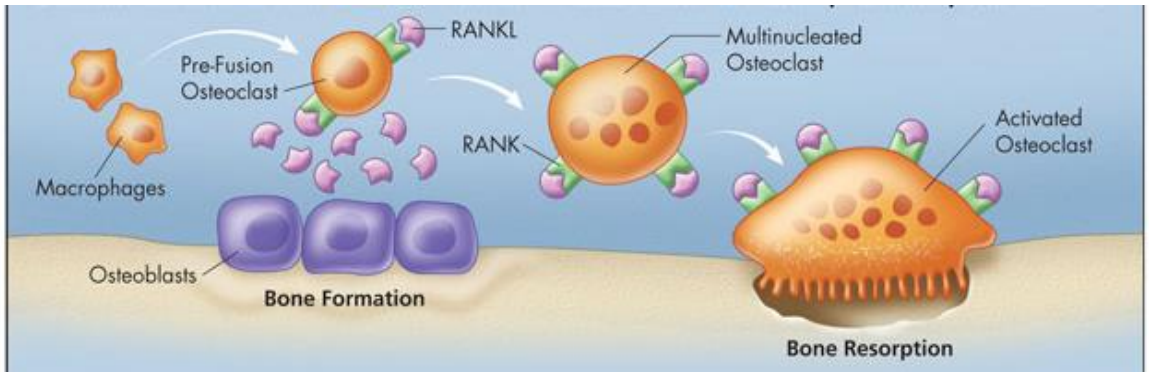

Figure 3 – **RANK/RANKL signaling pathway representation and osteoclastogenesis**. Osteoblasts in bone produce receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL activates its receptor, RANK, which is expressed on the surface of pre-fusion osteoclasts, generating activated osteoclasts (Josse RG, 2009).

Physiological bone resorption is typically followed by formation through osteoblasts, permitting the essential replacement of old skeletal tissue with new. This relationship between osteoclast and osteoblast function is disrupted in RA, which is characterized by limited bone formation despite high resorptive activity. Thus, in the RA pathogenesis there is an imbalance between pro- and anti-inflammatory mediators and of bone resorption and formation due to the predominance of the pro-inflammatory signals and bone resorption.

In RA the mechanisms of neutrophil activation, recruitment and apoptosis are altered. Circulating neutrophils from RA patients seem to have decreased levels of spontaneous apoptosis (Weinmann P et al., 2007). Herein, there is an impairment of neutrophil clearance and the ingestion of these cellular by macrophages induces production of pro-inflammatory cytokines, thus amplifying the inflammatory scenario (Sweeney SE, Firestein GS, 2004).

Activated neutrophils specifically play an important role in the onset and perpetuation of RA, not only as pro-inflammatory cytokine (such as IL-1β and TNF) and chemokines-producing cells, but also for being responsible for the release of high amounts of reactive oxygen species (ROS) and destructive enzymes, such as metalloproteinases (MMPs), contributing to inflammation and joint erosions (Nathan C et al., 2006; Scapini P et al., 2000; Cascao R et al., 2010).

B cells contribute to RA pathogenesis not only through antigen presentation, but also through the production of antibodies, autoantibodies and cytokines (Smolen JS et al., 2007). B cells, once differentiated as plasma cells, are able to produce and release autoantibodies such as Rheumatoid factor (RF) and Anti-citrullinated protein antibodies (ACPA), which can occur even before the clinically onset of disease (Edwards CJ et al., 2006).

These autoantibodies can form larger immune complexes which can activate

inflammatory cells (macrophages), and further stimulate the production and release of several pro-inflammatory cytokines (including TNF, IL-1β and IL-6) in synovial tissue (Kinne RW et al., 2007), that exacerbate the inflammatory process as previously described (Smolen JS et al., 2007). Interestingly, it has been evidenced that a decreased B cell count, corresponding to lower memory B cell numbers, in the periphery, is associated with arthritis development (Lubbers J et al., 2015).

Recent evidences have indicated clear involvement of Th17 and regulatory T (Treg) cells in the RA pathogenesis (Alzabin S et al., 2011; Wehrens EJ et al., 2013), and a relatively small contribution of Th1 in the global context of this disease (Boissier MC et al., 1995). Of note, Th2 cells seem to only have a modest contribution for RA pathogenesis as can be appreciated by the low expression level of their cytokines in the synovial tissue (Woods JM et al., 1997; Morita Y et al., 1998).

Previous studies have implicated IL-17-producing T cells (Th17) as important effectors in RA pathogenesis due to the overexpression of IL-17 in the synovial fluid, which is associated with an aggravation of inflammation and joint damage (Lubberts E et al., 2005; Chabaud M et al., 1999). Concretely, this cytokine is involved in overexpression of other cytokines, cartilage-destructive enzymes and also expression of bone destruction-related mediators, such as RANKL (Mclnnes IB et al., 2007; Choy EH et al., 2001). In fact, the percentage of Th17 cells is increased in RA synovial fluid in comparison with RA or normal peripheral blood (Shahrara S et al., 2008). Previous reports have demonstrated that IL-6 and IL-1β secreted by self-reactive T cell-stimulated APCs, are able to induce the differentiation of naïve self-reactive T cells into Th17 cells (Hirota K et al., 2007; Chung Y et al., 2009; Kryczek I et al., 2007), via 'retinoic acid receptor-related orphan receptor gamma T' (RORgT). In fact, cytokines that support Th17 differentiation, suppress Treg cells polarization, and consequently shift T cell homeostasis towards inflammation.

Regulatory T (Treg) cells have been detected in the blood and in synovium of RA patients with active disease and particularly in synovial fluid, but they seem to have impaired regulatory function (Ehrenstein MR et al., 2004). Contrarily to Th1, Th2 and Th17 cells, Treg cells are characterized by low proliferative capacity upon triggering the T cell receptor (TCR) and by their ability to suppress CD4+ and CD8+ T-cell immune responses (Suri-Payer E et al., 1998). Thus, to prevent overshooting T-cell activation, a means of T-cell control is achieved by Treg cells, a population of CD4+CD25+ cells expressing the transcription factor 'forkhead box protein 3' (Foxp3) that effectively suppresses T-cell

activation. However, several studies have pointed out that Treg cells are deficient in RA (Ehrenstein MR et al., 2004) suggesting that in fact a breakdown of Treg-mediated peripheral tolerance may have occurred (Suri-Payer E et al., 1998; van Amelsfort JM et al., 2004; Moradi B et al., 2014).

Accordingly, an imbalance between the T regulatory cells (Treg) and the T helper cells (Th17) in RA patients has been reported and associated with the disease development (Niu Q et al., 2012).

1.4. TREATMENT OPTIONS

The treatment goal in RA is remission (van Tuyl LH et al., 2009; van Tuyl LH et al, 2010) which is achieved in 20%-30% of patients if early and optimally treated (Lindqvist E et al., 2002).

In the last decades, there was a great improvement in RA therapeutic approaches, however despite all available treatment options, RA remains an incurable, progressive and debilitating disease (Burmester GR et al., 2017). Glucocorticoids (GCs), conventional and targeted synthetic disease modifying anti-rheumatic drugs (DMARDs) and biologic DMARDs, represent the most commonly used therapeutic strategies in the treatment of RA (van Vollenhoven RF et al., 2009; Yang M, et al., 2017).

GCs such as prednisolone and dexamethasone are suppressors of the inflammatory response and, consequently of the pain and swelling (Gaffo A et al., 2006), reducing synovitis in the short-term and decreasing joint damage in the long-term (Kirwan JR et al., 2007). GCs are widely used in treating active RA but side effects such as immunosuppression, osteoporosis, hyperglycaemia and hypertension associated with the dosage cannot be ignored during long-term therapy (Polido-Pereira J et al., 2011; van Vollenhoven RF et al., 2009). Currently, GCs are used in an initial phase of the disease and are useful to control symptoms immediately after diagnosis, until slower-action DMARDs can start to have an effect (Smolen JS et al., 2017).

Conventional synthetic DMARDs (csDMARDs) such as methotrexate (MTX), hydroxychloroquine, sulfasalazine and leflunomide are the most used drugs to efficiently treat RA patients (Donahue KE et al., 2008). csDMARDs can slowly (1 up to 6 months) relieve joint damage and control the disease progression, reducing synovitis and systemic

inflammation and improving function (Gaffo A et al., 2006; Cronstein B et al., 2005). MTX is the most commonly used csDMARD agent, and it is effective on standard clinical measures of disease activity (Strand V et al., 1999) cost-effective and comparatively well tolerated. csDMARDs have been shown to provide a more favourable outcome in patients. However, approximately 30% of the patients are either non-responsive to csDMARDs or lose response over time or experience adverse events following treatment (Cash JM et al., 1994; Nagashima M et al., 2006; Keystone EC et al., 1999; Rau R, 2010).

Biologic DMARD agents (bDMARDs) have been developed to target a specific molecule and/or pathway and are used when arthritis is uncontrolled or toxic effects arise with csDMARDs. Among them, inhibition of TNF, IL-1β, IL-6 receptor, T-cell costimulation blockade, and B-cell depletion have appeared as an alternative to csDMARDs (Nam JL et al., 2017). Evidences suggest that biologic agents are highly effective, although 30% of patients still show low efficacy. In addition, various problems are associated with current biological therapies, since they must be administered parenterally, have adverse events and high costs, as they are considerably more expensive than standard treatment options (Koenders MI et al., 2015; van Vollenhoven RF et al., 2009).

Lastly, targeted synthetic DMARDs (tsDMARDs) which are also developed to target a specific molecule/pathway, but unlike the biologics these are small molecules. Tofacitinib and baricitinib are two examples of small-molecule Janus kinase (JAK) inhibitors (Smolen JS et al., 2016; Nam JL et al., 2017). tsDMARDs are recommended only after failure to meet the treatment goal with MTX (Smolen JS et al., 2017) or more than one bDMARD.

Unfortunately, the most recent and innovate therapies are highly expensive limiting the access to the standard of care. Moreover, many of these therapies are only effective in controlling inflammation or protecting bone damage (Fonseca JE et al., 2009; Joosten LA et al., 1999), which is the major burden in RA patients. Consequently, the development of less expensive small molecules for the treatment of RA which are safe and effective in the control of both inflammation and bone damage, is still a medical unmet need.

2. CELASTROL

2.1. CELASTROL

The growing need for safe therapies led to the discovery of a Chinese herb, the *Tripterygium wilfordii*, which has been used for centuries in the chinese medicine to treat inflammation and autoimmune related diseases including RA (Sassa H et al., 1990; Allison AC et al., 2001; Kim DH et al., 2009; Jaquet V et al., 2011; Venkatesha SH et al., 2011; Brinker AM et al., 2007; Lipsky PE and Tao XL, 1997; Qiu D and Kao PN, 2003; Canter PH et al., 2006). Extracts from *Tripterygium wilfordii* have already been used in RA patients (Tao X et al., 2002; Goldbach-Mansky R et al., 2009; Jiao J et al., 2012; Cibere J et al., 2003; Lv QW et al., 2015; Wu YJ et al., 2001; He WZ et al., 2014), reducing disease activity as effectively as csDMARDs (Liu Y et al., 2013; Wang HL et al., 2016). However, despite its potencial clinical usefulness, contradictory data regarding efficacy and safety has been published (Canter PH et al., 2006; Cameron M et al., 2011; Jiang Q et al., 2009; Qian SZ et al., 1987; Matlin SA et al., 1993; Yuan YY et al., 1995). Of note, herbal extracts exhibit a high variability in the concentration/quality of their bioactive constituents and the products used for extraction methods might cause tolerability problems, thus, the use of a purified component isolated from *Tripterygium wilfordii* that possesses the disease-modulating attribute of the natural plant extract may circumvent these limitations (Venkatesha SH et al., 2011).

Celastrol, is a bioactive of *Tripterygium wilfordii* that has already shown to possess potent anti-inflammatory, anti-tumoral and neuroprotective properties in *in vitro* and in animals models of cancer (Yu X et al., 2015; Li H et al., 2010; Zhao F et al., 2010; Zhou GS et al., 2011a; Abbas S et al., 2007; Lee JH et al., 2006; Davenport A et al., 2010), inflammatory (Ding Q et al., 2013; Kim DY et al., 2009; Kim DH et al., 2009; Shaker ME et al., 2014; Pinna GF et al., 2004) and neurodegenerative diseases (Paris D et al., 2010; Choi BS et al., 2014). These significant therapeutic properties of celastrol support the growing interest on treating patients with celastrol.

2.2. CELASTROL IN ARTHRITIS

The anti-inflammatory properties of celastrol can be mainly attributed to the regulation of cytokine and chemokine production (Kim DH et al., 2009; Lee JH et al., 2006; Venkatesha SH et al., 2011; Venkatesha SH et al., 2012; Lee JY et al., 2015; Cascão R et al., 2012; Cascão R et al., 2015), the modulation of inflammatory cell functions (Li G, Liu D et al, 2013; Li G et al., 2012; Li G et al., 2013; Yu Y et al., 2015; Astry B et al., 2015; Cascão R et al., 2015), osteoclast modulation and bone damage control (Idris AI et al., 2010; Nanjundaiah SM et al., 2012; Gan K et al., 2015; Cascão R et al., 2015), mostly due to its ability to downregulate the NF-kB pathway.

Using an adjuvant-induced rat model of arthritis (AIA), several studies have reported the anti-inflammatory and bone protective effects of celastrol via the inhibition of pro-inflammatory cytokines (such as TNF, IL-1β (Venkatesha SH et al., 2012; Cascão R et al., 2012; Li H et al., 2008; Astry B et al., 2015), IL-17, IL-6 and IL-8 (Nanjundaiah SM et al., 2012; Venkatesha SH et al., 2011)) and the levels of chemokines (such as the 'regulated upon activation, normally T cell expressed and secreted' (RANTES) and monocyte chemotactic protein 1 (MCP-1)) that mediate cellular infiltration into the joints (Venkatesha SH et al., 2012). Celastrol also reduces the number of Th17 cells and conversely, significantly increases Treg cells in the synovial tissue, promoting a restore of Th17/Treg balance (Astry B et al., 2015). Moreover, a significant inhibitory effect on antibody response by reducing serum levels of ACPA antibodies of the IgG class has also been observed in the same rat model upon celastrol adiminstration (Venkatesha SH et al., 2011). Using the same model of arthritis (AIA) celastrol has also shown a significant inhibitory effect on inflammatory arthritis and on the reduction of bone and cartilage damage by suppressing mediators of osteoclastic bone remodeling, RANKL expression and osteoclast numbers (Nanjundaiah SM et al., 2012). Also, celastrol can protect chondrocytes by downregulating the expression of metalloproteinases, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein (Ding Q et al., 2013).

Other studies using celastrol have also reported beneficial effects in various models of inflammation, in addition to AIA, such as in the collagen-induced arthritis (CIA) model, where it has also been observed that celastrol suppresses arthritis and bone damage as it is able directly inhibit osteoclast differentiation/formation (osteoclastogenesis) and function and consequently prevent bone destruction (Gan K et al., 2015; Nanjundaiah SM et al.,

2012). It has been shown in the joints of CIA mice and in RAW264.7 macrophagic murine cell line, that celastrol inhibits the formation of the osteoclasts cells and reduces the expression of osteoclastic genes and transcriptional factors beyond the bone-resorbing activity (Gan K et al., 2015).

It has also been described in cells isolated from RA patients and cultured *in vitro*, that celastrol can normalize synovial features inhibiting human RA fibroblast-like synoviocytes (FLSs) proliferation (Xu Z et al., 2013) and their migration and invasion (Li G et al., 2012; Li G et al, 2013; Li G, Liu D et al, 2013), possibly through suppression of Fas receptor (FasR) (also known as CD95; involved in cell apoptosis) (Xu Z et al., 2013), MMP-9 expression (responsible for bone and cartilage matrix degradation) (Li G et al., 2012; Li G et al, 2013), CXC chemokine receptor 4 (CXCR4) (involved in invasion processes) and hypoxia-inducible factor-1α (HIF-1α) expression (regulator in the cellular response to hypoxic conditions) (Li G, Liu D et al, 2013), respectively. Additionaly, it has been shown that celastrol specifically impairs the development of B cells in peripheral blood (Kusy S et al., 2012).

Based on previous data from our group showing that IL-1β plays an important role since the early phase of RA (Cascão R, Polido-Pereira J et al., 2012; Cascão R et al., 2010) and that pathways regulating this cytokine, together with TNF, can constitute promising combined therapeutic targets for RA, we have performed an *in vitro* drug screening (Figueiredo et al., ?) for compounds that simultaneously inhibit IL-1β and TNF production (Cascão R et al., 2012) and have identified celastrol as a promising therapeutic candidate.

Using the AIA rat model we have also demonstrated that this compound has significant anti-inflammatory and anti-proliferative properties through suppressing joint inflammation (ankle swelling, joint inflammatory cell infiltration and proliferation) (Cascão R et al., 2012) as well as reducing synovial infiltration of CD3+ T cells, CD19+ B cells, CD163+ macrophages, and most importantly, CD68+ macrophages (a biomarker of drug efficacy in RA) (Cascão R et al., 2015). Importantly, we have also revealed that celastrol has bone protective effects in the AIA rat model, as it is able to decrease the number of osteoclasts and osteoblasts present in arthritic joints and reduce local bone erosions, systemic bone loss and microarchitecture degradation (Cascão R et al., 2017).

Recently our own data showed *in vivo* in the same animal model, that the doses of 2,5 and 5μg/g/day are effective and non-toxic in the treatment of arthritis. However, lower concentrations immediately lose efficacy and higher concentrations show signs of toxicity (*our unpublished data*). These results are crucial for future experiments in order to

determine the maximum safe dose and pharmacokinetics in healthy rats, and predict the first-in-human dose in future experiments.

Altogether, these data showing that celastrol can effectively control the arthritis progression due to suppress not only the inflammation mediators but also to prevent cartilage damage and bone resorption, suggest that this compound constitute a promising candidate to the therapeutic targets of arthritis and subsequently, a new and suitable treatment option for RA disease.

CHAPTER II – AIMS

Celastrol has shown inhibitory effects in inflammation as well as in cartilage and bone damage *in vivo* and *in vitro*. Thus, this compound can constitute a promising candidate in the treatment of RA. Based on previous data about celastrol effects and considering the unmet medical need in RA therapeutic options, we hypothesize that celastrol is able to restore bone and inflammatory markers to basal levels and counteract joint infiltrating immune cells in RA patients. Therefore, we find convenient and appropriate to investigate the effect of celastrol on several leukocyte cell populations such as monocytes, granulocytes and lymphocytes as well as their subsets, on peripheral blood samples collected from RA patients.

The goal of this project is to analyze the **effect of celastrol** with or without **LPS stimulation** on **primary human leukocytes** (such as **monocytes, granulocytes, T cells and B cells** and their **subsets**) **activation**, **maturation**, **survival** and **apoptosis** using **peripheral blood samples** collected from **RA patients** in comparison with **healthy controls**. In order to accomplish this, after celastrol incubation, cell viability is assessed using alarmarBlue® by spectrophotometry assay, and an immunophenotyping characterization of monocytes, granulocytes, T and B cells is performed by Flow Cytometry.

The present project is innovative because it enables a very complete understanding underlying the effect of celastrol on primary immune cells.

CHAPTER III – MATERIALS AND METHODS

1. COMPOUNDS

Celastrol was purchased from Sigma (Missouri, USA), dissolved in ethanol 100% as solvent (absolute ethanol) (Qi X et al., 2014) and stored as aliquots (stock solution of 10 mg/mL) at –20ºC until used.

Purified lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma (Missouri, USA), dissolved in Phosphate Buffered Saline (PBS) 1x and stored as aliquots (1000 µg/mL) at -20ºC until used.

2. HUMAN SAMPLES

Peripheral blood samples were collected from RA patients (*n*=5) (Rheumatology Department, Hospital de Santa Maria, Lisbon) with active disease (disease score > 3.2) and healthy individuals (*n*=10) used as controls (**Table 1**). Patients cohort included RA patients who fulfilled the 2010 ACR/EULAR criteria for RA (Aletaha D et al., 2010) with more than 1 year of disease duration, RF and/or ACPA positivity (seropositive) and treated with methotrexate (MTX) ± prednisone (PDN) ≤ 7,5 mg/day ± NSAIDs.

The disease activity score using 28 joint counts (DAS28) were applied to all patients. This study was approved by the local ethics committee (Comissão de Ética do Hospital de Santa Maria, Lisbon, Portugal) and all patients and healthy donors signed an informed consent form. Patient care was conducted in accordance with standard clinical practice and the study was performed in accordance with the Declaration of Helsinki as amended in Fortaleza, Brazil (2013).

Blood was collected for tubes containing Sodium Heparin to proceed to the isolation of leukocytes. Additionally, blood were also collected for a vacutainer blood collection tube containing anticoagulant EDTA (BD Vacutainer®), to perform white blood cell (WBC) count using a pocH-100*iV Diff* ™ Automated Hematology Analyzer (Sysmex).

3. ISOLATION OF LEUKOCYTES FROM BLOOD

Blood was resuspended and incubated in an erythrocyte lysis buffer (Red Blood Cell (RBC) lysis buffer) at 1:10 dilution, for 10 min at room temperature (RT). After centrifugation at 300*g*, for 15 min at RT, the supernatant was discarded and the white blood cells (WBC) fraction (pellet) was collected. Cells were resuspended in RPMI 1640 (ThermoFisher Scientific) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) Penicillin-Streptomycin (Pen-Strep), 1% (v/v) Sodium Pyruvate, 1% (v/v) L-Glutamine, 1% (v/v) MEM Non-essential aminoacids, 1% (v/v) HEPES Buffer and 0,05 mM 2-mercaptoethanol (ThermoFisher Scientific). Viable peripheral WBCs were count using Trypan Blue dye (BioWhittaker™) on a Neubauer Chamber (hemocytometer).

4. *IN VITRO* TEST OF CELASTROL

4.1. OPTIMIZATION OF EXPERIMENTAL CONDITIONS

Cells were seeded into 24-well plates at a density of $1x10^6$ cells per well, incubated with a range of celastrol concentrations (0,001 µM, 0,01 µM, 0,05 µM, 0,1 µM, 0,3 µM, 0,5 µM and 1 µM) and stimulated with LPS (2 µg/mL, 5 µg/mL and 10 µg/mL), for different incubation times (0h, 4h, 16h and 24h) in a 5% CO_2 enriched atmosphere at 37ºC and protected from light exposure. Four experimental conditions were studied: Media + Ethanol 100% (solvent); Media + Ethanol 100% + LPS; Media + Ethanol 100% + Celastrol and Media + Ethanol 100% + Celastrol + LPS. The condition Media + Ethanol 100% was used as a control condition. Accordingly, one condition studied either at 0h incubation time as in the incubation time to be tested, in particular, Media (Media Only) was used as control condition. Cell viability was assessed using alamarBlue® cell viability test by spectrophotometry and 7-Amino-actinomycin D (7-AAD) Apoptosis Staining by Flow Cytometry, to subsequently determine the best compounds concentrations and incubation time to use. Triplicates of each experimental condition were used.

The selection of the range of concentrations to be tested were based in *in vitro* experiments and in *in vivo* pharmacokinetics tests of celastrol reported by Zhang J et al. (Zhang J et al., 2012).

4.2. OPTIMIZED IN VITRO TEST OF CELASTROL

Cells were seeded into 24-well plates at a density of $1x10^6$ cells per well, incubated with 0,3 μM of celastrol and stimulated with 10 μg/mL LPS for 4h in a 5% CO_2 enriched atmosphere at 37ºC and protected from light exposure. Four experimental conditions were used: Ethanol 100% (solvent) + Media; Celastrol 0,3 μM + Media; LPS 10 μg/mL + Media and Celastrol 0,3 μM + LPS 10 μg/mL + Media. Duplicates of each condition were used to evaluate cell viability using alamarBlue® cell viability test by spectrophotometry assay. The immunophenotyping characterization of WBC subpopulations, including monocytes, granulocytes, T and B lymphocytes from each condition was performed by Flow Cytometry.

5. FLOW CYTOMETRY

5.1. ANTIBODIES

Antibody titration was performed using the concentration indicated in the respective data sheet (1:20 ratio) and two concentrations below (1:40 and 1:100 ratio) directly stained on 100 μL peripheral blood cells of healthy controls, in order to define the concentration that better stain and present well defined cell subsets with the brightest signal.

Immunophenotyping characterization of monocytes, granulocytes, T and B cells and their subsets was performed in primary human leukocytes isolated from peripheral blood samples of healthy controls and chronic RA patients after incubation.

For B cell analysis, combinations of anti-CD19 PerCP-Cy5.5, anti-IgD PE-Cy7, anti-CD27 eFluor450, anti-CD38 APC-eFluor 780, anti-BAFF-R PE, anti-FcgRIIB (CD32) APC, anti-HLA-DR APC, anti-CD21 PE, anti-CD95 APC and anti-RANKL PE were used.

T cells were studied with combinations of anti-CD3 APC, anti-CD4 PerCP-Cy5.5, anti-CD8 PE, anti-CD45RO APC-eFluor 780, anti-CD40L PE-Cy7, anti-HLA-DR eFluor 450, anti-CD25 PE-Cy7, anti-Foxp3 eFluor 450, anti-RORgT PE, anti-CCR6 PE, anti-CXCR3 PE-Cy7 and anti-CCR7 Pacific Blue.

For monocyte analysis, combinations of anti-CD14 PerCP-Cy5.5, anti-CD3 APC, anti-CD86 PE, anti-HLA-DR eFluor 450, anti-CD16 APC-Cy7, anti-CD115 PE-Cy7 and anti-

RANK PE were used.

For granulocyte analysis, combinations of anti-CD66b PE-Cy7, anti-CD11b PerCP-Cy5.5, anti-CD16 APC-Cy7, anti-CXCR2 PE, anti-CD62-L APC and anti-CD15 eFluor 450 were used.

Antibodies were purchase from eBioscience (USA), BD Pharmigen (USA), R&D Systems (United Kingdom), BioLegend (San Diego), Santa Cruz Biotechnology.

5.2. LEUKOCYTE STAINING – PREPARATION OF SAMPLES TO FLOW CYTOMETRY

After incubation with celastrol and LPS, cells were carefully ressuspended and transferred from the 24-well plates into to the cytometer tubes. After a centrifugation at 300*g* for 5 min at RT, supernatants from each tested condition were collected and stored at -80ºC for future enzyme linked immunosorbent assay (ELISA) quantifications. Then, cells were washed with PBS1x and afterwards, they were resuspended in PBS1x for antibody staining for flow cytometry.

5.2.1. *Cell surface staining protocol*

Cells were stained on ice for 15 minutes, with 9 different mixes of surface antibodies (**Appendix I**). Of note, the FVD FITC antibody was added to each Mix immediately prior to staining cells, to prevent any loss of staining intensity of the dead cells. After cell surface staining, cells were washed with PBS1x, ressuspended and centrifuged at 300*g* for 5 min at RT and protected from light exposure. The supernatant was discarded and then cells were ressuspended in 300 µL of PBS1x and stored at 4ºC protected from light exposure until analyzed by flow cytometry. Samples were analyzed using a BD LSRFortessa™ Cell Analyzer (BD Biosciences, New Jersey, USA) with BD FACSDiva™ v6.1 Software (BD Biosciences, San Jose, CA). The data collected were further analyzed using FlowJo 9.8.2 Software (Tree Star, Stanford University, USA).

5.2.2. Intracellular staining protocol

Cells (corresponding to Mix 5) were ressuspended in PBS1x (100 μL) and then, the intracellular staining protocol was performed as described in the "Staining Intracellular Antigens for Flow Cytometry" protocol (eBioscience, USA). Briefly, cells were fixed during 30 minutes on ice at 4ºC with Foxp3 Fixation/Permeabilization working solution, permeabilized with 1x Permeabilization Buffer and stained with the intracellular antibodies (Foxp3 and RORgT), for 30 minutes on ice at 4ºC in the dark. Finally, cells were washed twice with PBS1x, ressuspended and centrifuged at 300*g* for 5 min at RT, protected from light exposure. The supernatant was discarded and then, cells were prepared as samples to flow cytometry analysis. They were ressuspended in 300 μL of PBS1x and stored at 4ºC protected from light exposure until analyzed by flow cytometry, as above mentioned.

6. ALAMARBLUE® CELL VIABILITY ASSAY

After the incubation with celastrol and LPS, cell viability was assessed by alamarBlue® assay (ThermoFisher Scientific) in accordance with the manufacturer's instructions. Briefly, 10 % (v/v) of alamarBlue® reagent (resazurin) (100 μL) was added directly to the cells of each condition and incubated for 2 hours at 37ºC and 5% CO_2 in the dark. Fluorescence intensity was recorded with 570 nm excitation and 610 nm emission wavelengths, using a microplate reader Infinite M200 (Tecan, Switzerland).

In the assessment of cell viability for optimization of the celastrol and LPS doses and durantion of incubation, the percentage/number of viable cells was determined by comparing each fluorescence value with the fluorescence obtained at the incubation time of 0 h. For the remaining measurements, cell viability was determined by comparing fluorescence values with those corresponding to the media (control).

7. 7-AMINO-ACTINOMYCIN D (7-AAD) APOPTOSIS STAINING

Cell death was determined by 7-Amino-actinomycin D (7-AAD) Apoptosis Staining (ThermoFisher Scientific), according to the provider's instructions. The proportions of cells in either population undergoing apoptosis were detected by staining of apoptotic cells with 7-AAD. After the incubation time of WBCs in the optimization of experimental conditions, 5 µl of the ready-to-use reagent was added directly to cells of each condition and incubated for 5 minutes, to determine the best compounds concentrations and incubation time to use. Samples were then acquired with a BD Accuri™ Flow Cytometer (BD Biosciences). The data collected were further analyzed using FlowJo 9.8.2 Software (Tree Star, Stanford University, USA).

8. STATISTICAL ANALYSIS

The normality distribution was assessed by D'Agostino and Pearson omnibus normality test. For populations that did not follow a Gaussian distribution, the non-parametric Mann-Whitney and Wilcoxon's matched pairs signed rank tests were used. The Mann-Whitney test was used for comparisons between 2 independent groups and the Wilcoxon matched pairs test for comparisons between 2 paired groups. Statistical differences were determined with GraphPad Prism 6 Software (GraphPad, California, USA). Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values < 0,05.

CHAPTER IV – RESULTS

1. *IN VITRO* TEST OF CELASTROL

1.1. OPTIMIZATION OF EXPERIMENTAL CONDITIONS

We first proceeded to the optimization of experimental conditions. To this purpose, a group of healthy controls (*n*=4) was required to collect peripheral blood samples. Isolation of primary human leukocytes was performed and cells were incubated for 0h, 4h, 16h and 24h with a range of concentrations of celastrol (0,001 µM, 0,01 µM, 0,05 µM, 0,1 µM, 0,3 µM, 0,5 µM and 1 µM) and LPS (2 µg/mL, 5 µg/mL and 10 µg/mL). We assumed 100% of viability at 0h (control condition at baseline) and that cell viability reductions above 20% would be significant.

1.1.1. INCUBATION TIME OPTIMIZATION OF CELASTROL

Data from cell viability assay (**Figure 4**, showed the celastrol incubation during 24h was the incubation time which most affected the cell viability, specifically under the 0,3 µM, 0,5 µM and 1 µM concentrations of celastrol (**Figure 4C**) when comparing to the control condition baseline at 0h (Media Only at 0h). However, not exceeding the value that we defined as the cutoff (20% decrease in cell viability), nor being decreased relative to the cell viability of the control conditions at 24h (Media Only at 24h and Media + Ethanol at 24h). Of note, comparing cell viability under these celastrol concentrations (0,3 µM, 0,5 µM and 1 µM) to the cell viability baseline at 0h (control condition at 0h - Media Only at 0h), we noticed that celastrol already appears to slightly decrease cell viability after 24h of the incubation time, but still, with no marked toxicity (**Figure 4C** and **4D**). Regarding to the incubation time at 4h and 16h, it was not observed a decrease in the cell viability in all celastrol concentrations when comparing to cell viability of control conditions at 0h and 4h/16h, respectively (Media Only at 0h; Media Only at 4h/16h and Media + Ethanol at 4h/16h) (**Figure 4A**, **4B** and **4D**).

AlamarBlue ® viability assay – *Cell viability analysis*

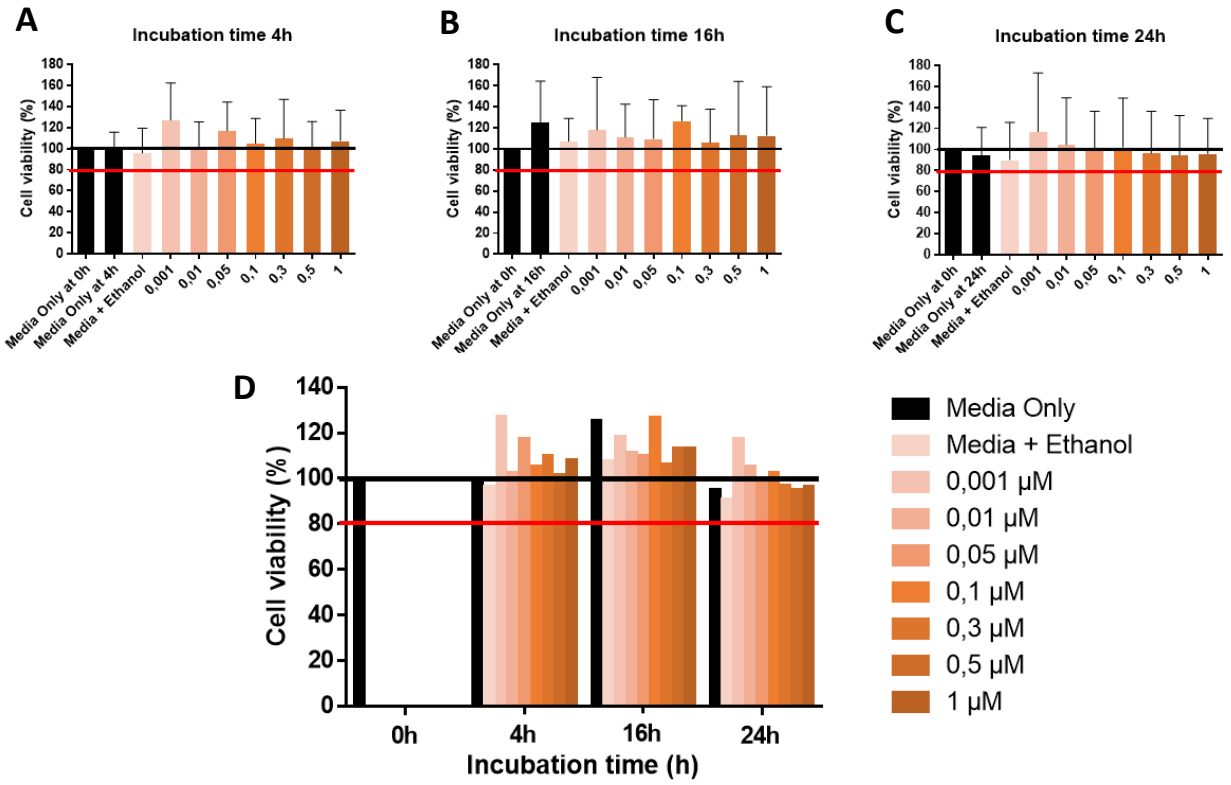

Figure 4 – **Incubation time optimization of celastrol in *in vitro*. Cell viability analysis by alarmarBlue® assay. (A)** Cell viability is not affected with a range concentrations of celastrol (0,001 µM - 1 µM) following 4h of incubation time. Healthy controls, *n*=4. **(B)** Cell viability is not affected with a range concentrations of celastrol (0,001 µM - 1 µM) following 16h of incubation time. Healthy controls, *n*=2. Instead, cell viability is increased after incubation of celastrol at both 4h **(A)** and 16h **(B)** incubation time. **(C)** Cell viability is not affected with a range concentrations of celastrol (0,001 µM - 1 µM) following 24h of incubation time. Healthy controls, *n*=2. However, slightly decrease of cell viability after 24h incubation time, when comparing to 4h and 16h, was observed, but sill, not exceeding the value that we defined as the cutoff (80%). **(D)** Incubation times tested (4h, 16h and 24h) do not seem affect cell viability when incubated with a varying concentrations of celastrol (0,001 µM - 1 µM), and therefore, not appearing to represent apparent toxicity. **Data from the alarmarBlue® cell viability assay.** Data are expressed as mean with standard deviation.

Flow cytometry analysis showed that both at 16h and 24h of incubation with celastrol led to a significantly decrease of monocytes viability, in all tested concentrations, comparing to the control condition baseline at 0h (Media Only at 0h) (**Figure 5v** and **5vi**). Moreover, lymphocytes viability also appeared to be affected either after 16h and 24h of incubation with celastrol (**Figure 5ii**). Contrarily, granulocytes viability was not impaired after 16h and 24h of incubation with celastrol, when comparing to the control condition baseline at 0h (Media Only at 0h) (**Figure 5viii** and **5ix**).

These data lead us to exclud the 16h and 24h of incubation from our experimental set-up and to select the incubation time of 4h.

Flow Cytometry – *Cell viability and subpopulation percentage analysis*

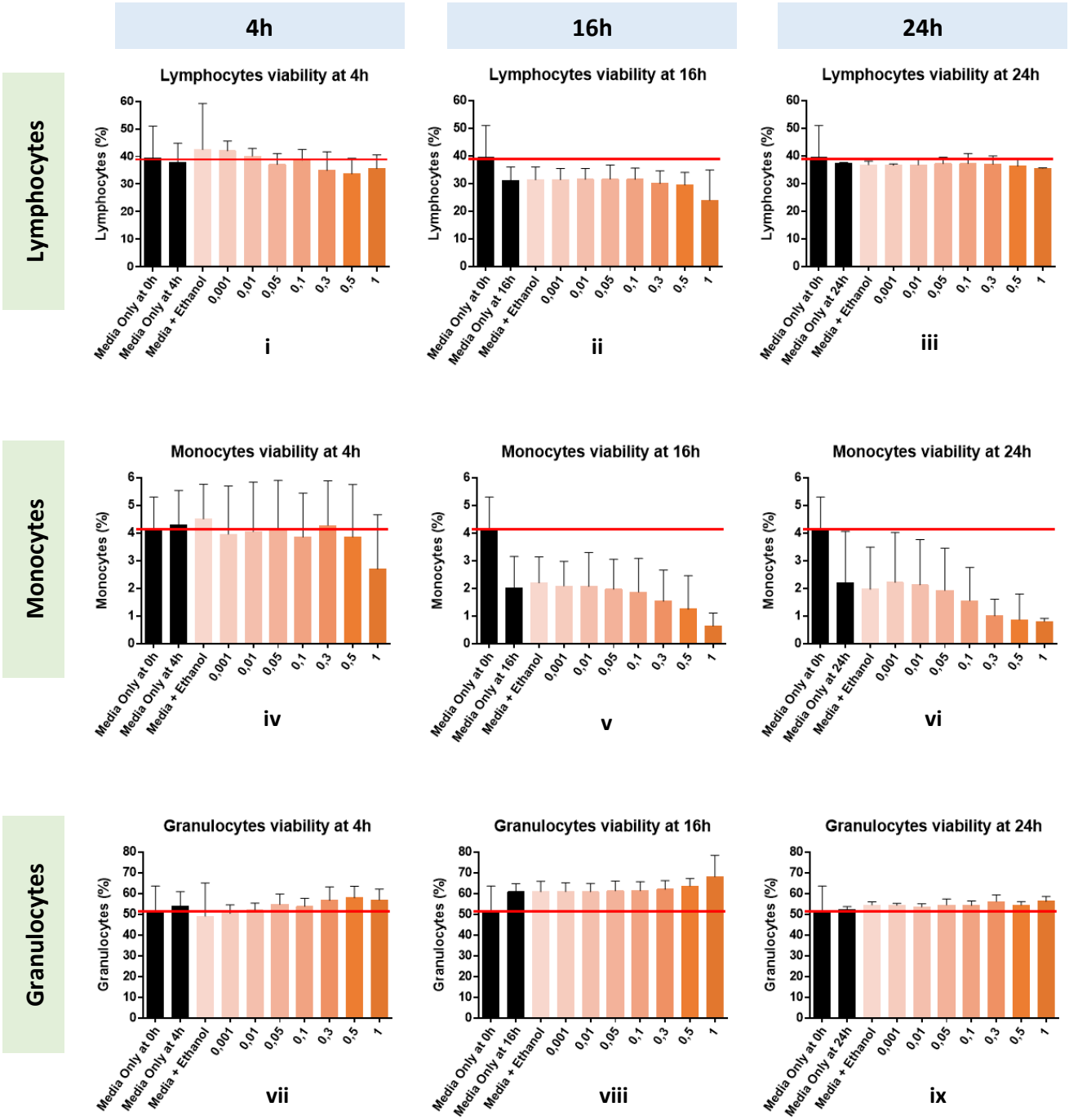

Figure 5 – **Incubation time optimization of celastrol in *in vitro*. Cell viability and subpopulation percentage analysis by Flow Cytometry.** Data from the Flow Cytometry analysis. Data are expressed as mean with standard deviation.

1.1.2. CONCENTRATION OPTIMIZATION OF CELASTROL

After 4h of incubation with celastrol, we particularly found that the 0,1 µM and 0,5 µM concentrations of celastrol slightly reduced the monocytes viability, although, a more pronounced reduction was observed using the highest dose of celastrol (1 µM), when comparing to the control condition baseline at 0h (Media Only at 0h) (**Figure 5iv**). In addition, lymphocytes viability at 4h was essentially affected with 0,5 µM and 1 µM concentrations of celastrol, when comparing to the control condition baseline at 0h (Media Only at 0h), but with no evidence of significant reduction in viability (**Figure 5i**). Granulocytes viability appeared increased following 4h of incubation with celastrol (0,001 µM - 1 µM), when comparing to the control condition baseline at 0h (Media Only at 0h) (**Figure 5vii**). As noted, the highest concentration of celastrol appears with apparent toxicity on monocytes and lymphocytes after 4h of incubation.

In order to evaluate and select the best celastrol concentration (0,001 µM, 0,01 µM, 0,05 µM, 0,1 µM, 0,3 µM, 0,5 µM or 1 µM) after 4h of incubation with this compound, a 7-AAD Apoptosis Staining was performed by Flow Cytometry to analyze the cell death in the different subpopulations (**Figure 6**).

Flow Cytometry – *Cell viability and subpopulation percentage analysis*

7-AAD Apoptosis Staining – *Subpopulations cell death analysis*

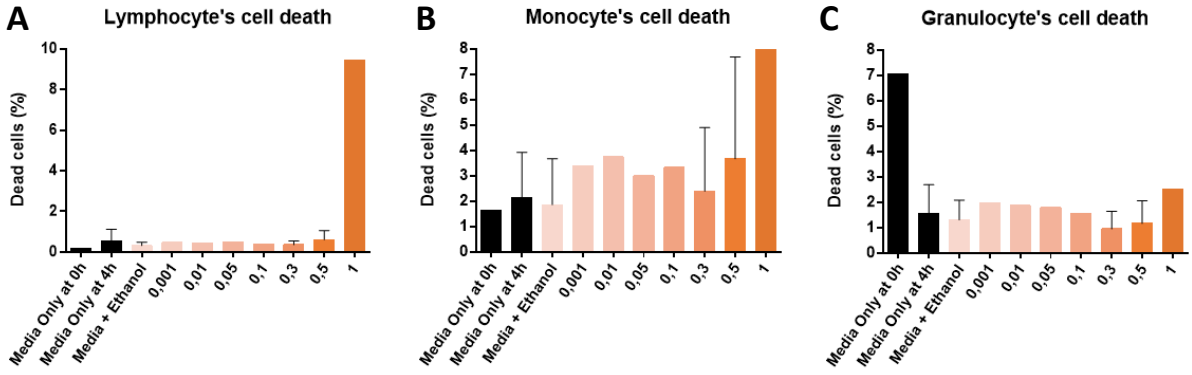

Figure 6 – Concentration optimization of celastrol in *in vitro* under incubation time of 4h. Analysis of subpopulations cell death by 7-AAD Apoptosis Staining and Flow Cytometry. Data from the Flow Cytometry analysis. Data are expressed as mean with standard deviation.

We observed that the highest dose of celastrol (1 µM) increased cell death in the lymphocytic population (**Figure 6A**). Concerning to the granulocytes, we observed that cell death was more pronounced at the highest dose of celastrol (**Figure 6C**). Concordantly, the highest concentration of celastrol also showed a marked increase in monocyte's cell (**Figure 6B**). Also, the concentration of 0,5 µM showed a slight decrease in cell viability, specialy in monocytes.

These data lead us to exclud the highest concentration of celastrol (1 µM) from our experimental set-up and to select 0,3 µM and 0,5 µM of celastrol to be tested in following experiments. According to these data, we selected the 0,3 µM and 0,5 µM of celastrol, to be further tested with a range of LPS stimulus concentration (2 µg/mL, 5 µg/mL and 10 µg/mL) (**Figure 7**).

1.1.3. CONCENTRATION OPTIMIZATION OF LPS

We next proceeded to the alamarBlue® viability assay and we found that the Celastrol 0,5 µM + LPS 2 µg/mL , Celastrol 0,5 µM + LPS 5 µg/mL and Celastrol 0,5 µM + LPS 10 µg/mL incubation during 4h were the conditions which most affected the cell viability, exceeding the value that we defined as the cutoff (20% decrease in cell viability) of cell viability (**Figure 7**). Regarding the remaining conditions, we observed that Celastrol 0,3 µM + LPS 2 µg/mL was the only condition with both stimuli that did not affect cell viability; in addition, none of the tested concentrations of Celastrol nor LPS markedly reduced cell viability.

AlamarBlue ® viability assay – *Cell viability analysis*

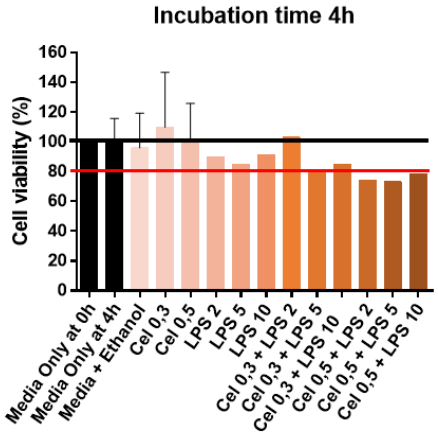

Figure 7 – Concentration optimization of celastrol with LPS in *in vitro* under incubation time of 4h. Cell viability analysis by alarmarBlue® assay. Data from the alarmarBlue® cell viability assay. Data are expressed as mean with standard deviation.

To assess how these conditions particularly could affect the viability of the subpopulations, we then performed a Flow Cytometry analysis (**Figure 8**).

Flow Cytometry – *Cell viability and subpopulation percentage analysis*

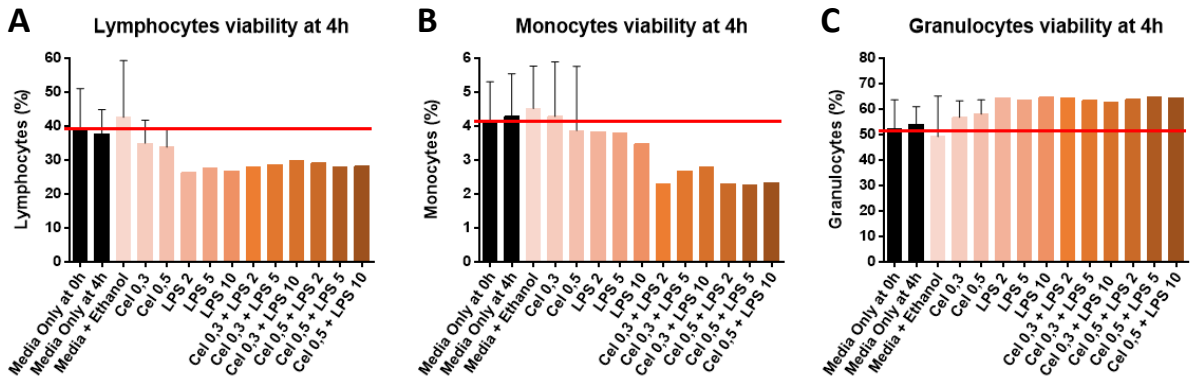

Figure 8 – Concentration optimization of celastrol with LPS in *in vitro* under incubation time of 4h. Cell viability and subpopulation percentage analysis by Flow Cytometry. Data from the Flow Cytometry analysis. Data are expressed as mean with standard deviation.

Incubation for 4h with Celastrol 0,3 µM + LPS 10 µg/mL was the condition with both stimuli that affected less both the lymphocytes (**Figure 8A**) and monocytes viability (**Figure 8B**). Moreover, also in lymphocytes and monocytes, it was observed that Celastrol 0,3 µM appeared to affect less and increase, respectively, the cell viability of these populations. Regarding to the effect of the isolated concentration of LPS on the subpopulations, we found that, while in the lymphocytes the LPS 5 µg/mL condition seemed to be those which affected less the cell viability, in the monocytes it was the LPS 2 µg/mL condition that seemed to affect less the cell viability. Concerning to the granulocytes, we found that none of the tested conditions appeared to affect the viability (**Figure 8C**).

We finally performed a 7-AAD Apoptosis Staining to analyze the cell death of each subpopulations by Flow Cytometry (**Figure 9**).

Flow Cytometry – *Cell viability and subpopulation percentage analysis*

7-AAD Apoptosis Staining – *Subpopulations cell death analysis*

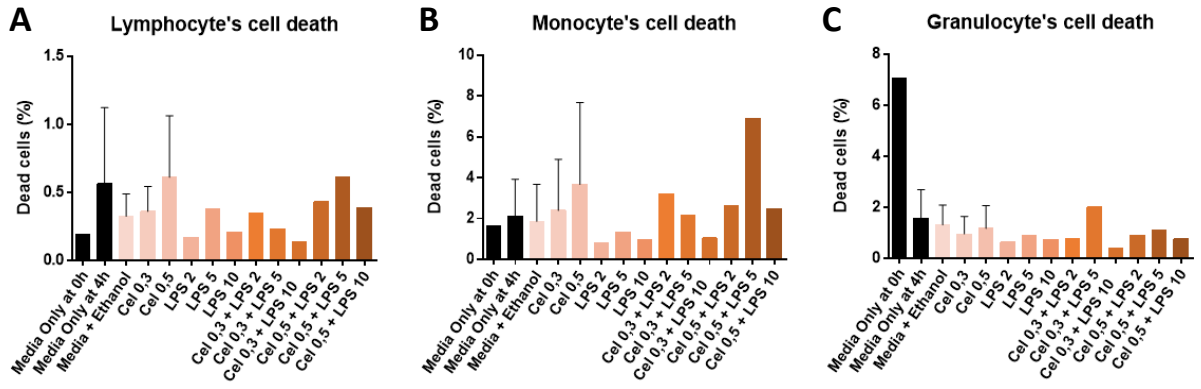

Figure 9 – **Concentration optimization of celastrol with LPS in *in vitro* under incubation time of 4h. Analysis of subpopulations cell death by 7-AAD Apoptosis Staining and Flow Cytometry. Data from the Flow Cytometry analysis.** Data are expressed as mean with standard deviation.

Among the tested concentrations, we found that incubation for 4h with Celastrol 0,3 µM + LPS 10 µg/mL was the condition that showed less lymphocyte's, monocyte's and granulocyte's cell death, appearing to be the best experimental condition to be tested in the following experiments (**Figure 9**).

2. HUMAN SAMPLES

2.1. CHARACTERIZATION OF PATIENTS COHORT

A group of RA patients (*n*=5) with active disease (disease score > 3.2) and with more than 1 year of disease duration (Aletaha D et al., 2010), was included in this study. All RA patients were under MTX treatment and had a mean age of 74 ± 5 years old, all female, 80% were RF positive, 40% were ACPA positive and DAS28 score was 4.5 ± 1.3. Additionally, blood samples were collected from healthy donors (*n*=10) for comparison. The clinical information from all patients and healthy controls included in this study is indicated in **Table 1**.

Table 1 - Clinical characterization about healthy controls and chronic RA patients.

	Controls (*n* = 10)	**Chronic RA patients (*n* = 5)**
***Age** (years)*	39 ± 5	74 ± 5*
***Sex** (% female)*	70	100
***Disease duration** (years)*	NA	10 ± 13
***RF-positive** (%)*	ND	80
***ACPA-positive** (%)*	ND	40
***CRP** (mg/dL)*	ND	1.6 ± 1.3
***ESR** (mm/1st hour)*	ND	36 ± 11
Patient VAS	NA	51 ± 17
DAS28	NA	4.5 ± 1.3
Tender joints	NA	4 ± 4
Swollen joints	NA	4 ± 4
Concomitant treatment:		
MTX	NA	1 / 5
MTX + PDN	NA	1 / 5
MTX + DFZ	NA	2 / 5
MTX + PDN + SLZ	NA	1 / 5
No treatment	NA	0 / 5

RA – Rheumatoid Arthritis; **RF** – Rheumatoid Factor; **ACPA** – Anti-Citrullinated Protein Antibody; **CRP** – C-reactive protein; **ESR** – Erythrocyte Sedimentation Rate; **VAS** – Visual Analogue Scale; **DAS28** – Disease Activity Score of 28 joints; **MTX** – Methotrexate; **PDN** – Prednisone; **DFZ** – Deflazacort; **SLZ** – Salazopyrin; **ND** – not determined; **NA** – not applicable. Values are represented as mean ± standard deviation. *Differences were considered statistically significant for *p-values* < 0.05 according to the Mann-Whitney test.

3. FLOW CYTOMETRY

3.1. HEALTHY CONTROLS

3.1.1. MONOCYTES

❖ *Classification of monocyte subpopulations and the effect of celastrol*

A gate was defined around total monocytes in peripheral leukocytes, depending upon cell size (forward scatter) and granularity (side scatter). Total monocytes were identified based on the expression of the cell surface marker CD14, excluding CD3 expression, and gating out cell aggregates and dead cells through by FVD marker. Monocytes can be subdivided into three subpopulations based on their expression of CD14 and CD16 surface markers and classified as **classical** (CD14+CD16-), **intermediate** (CD14+CD16+) and **non-classical** ($CD14^{dim}CD16+$) **monocytes** (Cros J et al., 2010).

The frequency of monocyte subsets was determined according to this classification system, although in this thesis the graphs presented are only relative to total CD14+ monocyte cells, due to the higher number of acquired cells, contrary to the insufficient cell events acquired in the gate of subpopulations. Of note, the analysis of all monocyte subpopulations was excluded in order not to lead to wrong interpretations. However, the graphs related to monocyte subpopulations are annexed as Appendix (**Appendix II**).

Total CD14+ cells

The analysis of the frequency (%) of total CD14+ cells has revealed that statistically significant differences were found between the tested conditions in healthy controls.

Specifically, it was observed that Celastrol (p=0.0008) and Celastrol + LPS (p=0.0015) significantly increased the levels of total CD14+ cells in comparison to LPS and restored the levels to basal levels (**Figure 10**).

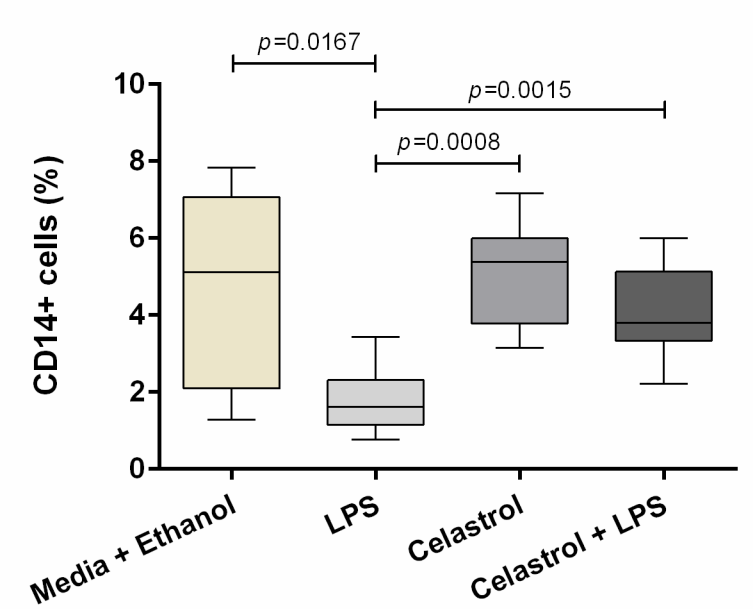

Figure 10 – Frequency of total CD14+ cells from healthy controls is significantly reduced with LPS condition in comparison to Media + Ethanol and significantly increased with Celastrol and Celastrol + LPS in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocyte cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

❖ *Monocyte markers and the effect of celastrol*

The expression of several cellular markers was analyzed to characterize the effect of celastrol on monocytes phenotype in healthy controls.

Classical activation monocyte markers (HLA-DR, CD16, CD86) were used and according to their function: HLA-DR (determined by % of positive cells and MFI values) - function as antigen-presenting cell (APC) as well as T-cell costimulation; CD16 (% and MFI) - activation and its phagocytic potential / the mediation of immunophagocytosis; CD86 (% and MFI) - monocyte-T cell costimulatory signalling required for T cell activation. CD115 (% and MFI) - receptor for macrophage-colony stimulating factor (M-CSF), essential for osteocastogenesis process; the receptor activator of nuclear factor-kB (NF-kB) (RANK) (% and MFI) - participates in osteoclast activation, differentiation and survival (osteoclastogenesis) and bone resorption.

CD16

Regarding CD16, it was observed that Celastrol (p=0.0080) significantly reduced the levels of CD16+ cells on total CD14+ cells in comparison to Media + Ethanol, and Celastrol + LPS significantly reduced the levels in comparison to Media + Ethanol (p<0.0001), LPS (p=0.0007) and Celastrol (p=0.0006) (**Figure 11**).

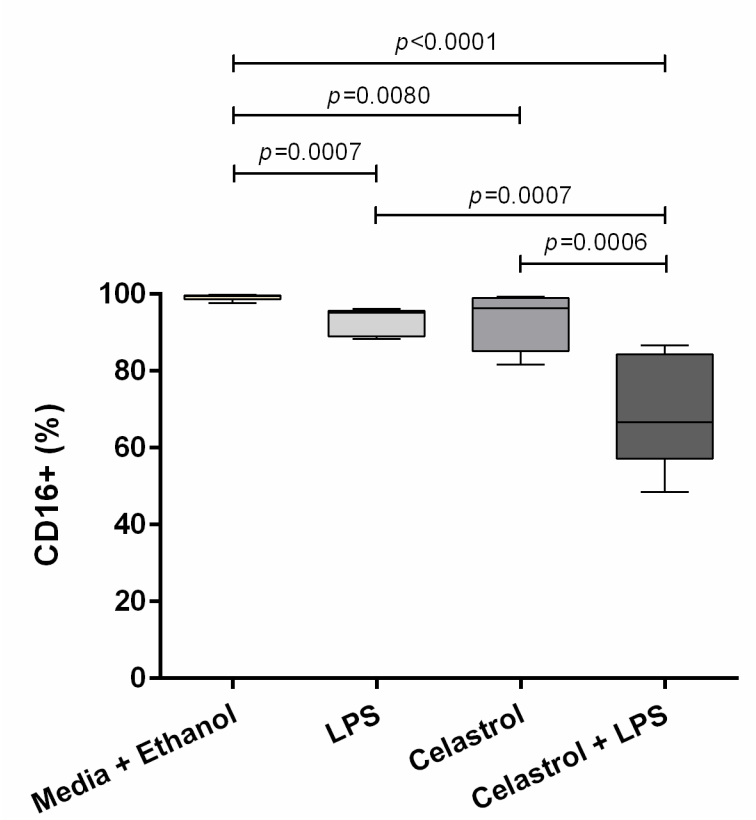

Figure 11 – Frequency of CD16+ cells on total CD14+ cells from healthy controls is significantly reduced with Celastrol and LPS conditions in comparison to Media + Ethanol, and significantly reduced with Celastrol + LPS condition in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocyte cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Furthermore, it was observed that Celastrol + LPS significantly reduced the expression of CD16 MFI values on total CD14+ cells in comparison to Media + Ethanol (p<0.0001), LPS (p=0.0225) and Celastrol (p=0.0003) (**Figure 12**).

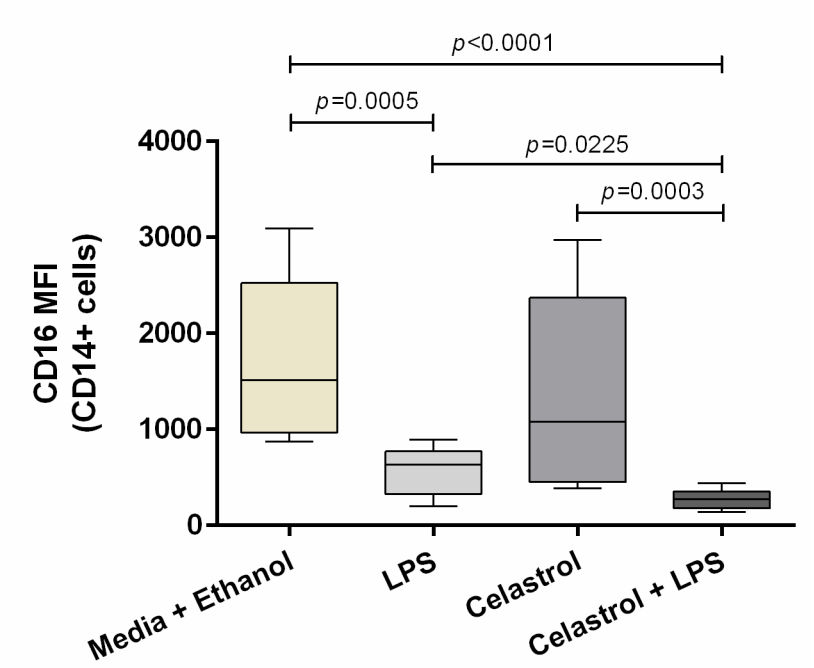

Figure 12 – Expression of CD16 (MFI) cell marker on total CD14+ cells from healthy controls is significantly reduced with LPS in comparison to Media + Ethanol, and also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

CD86

The analysis of CD86 on monocytes has revelead that Celastrol (p=0.0074) significantly increased the frequencies of CD86+ cells on total CD14+ cells in comparison to LPS and restored the frequencies to basal levels. Celastrol + LPS significantly reduced the frequencies in comparison to Media + Ethanol (p=0.0009) and Celastrol (p=0.0021) (**Figure 13**).

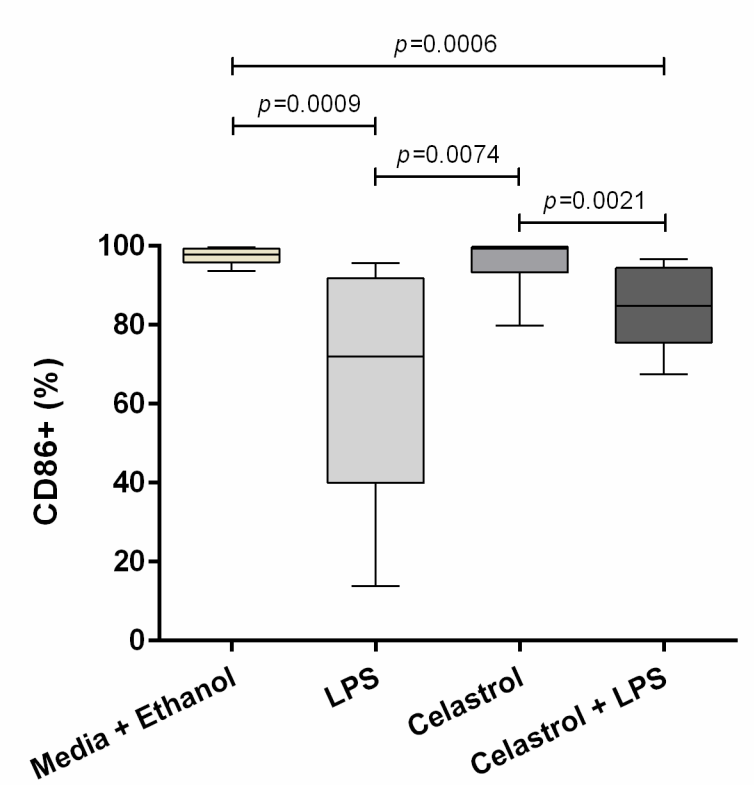

Figure 13 – Frequency of CD86+ cells on total CD14+ cells from healthy controls is significantly increased with Celastrol condition in comparison to LPS, and significantly reduced with Celastrol + LPS in comparison to Media + Ethanol and Celastrol. Data from flow cytometry analysis of peripheral blood monocyte cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Furthermore, significant differences were found in CD86 MFI values on total CD14+ cells.

Particularly, it was observed that Celastrol (p=0.0076) significantly increased the expression of CD86 MFI values on total CD14+ cells in comparison to LPS and restored the MFI values to basal levels. Celastrol + LPS significantly reduced the expression in comparison to Media + Ethanol (p=0.0007) and Celastrol (p=0.0101) (**Figure 14**).

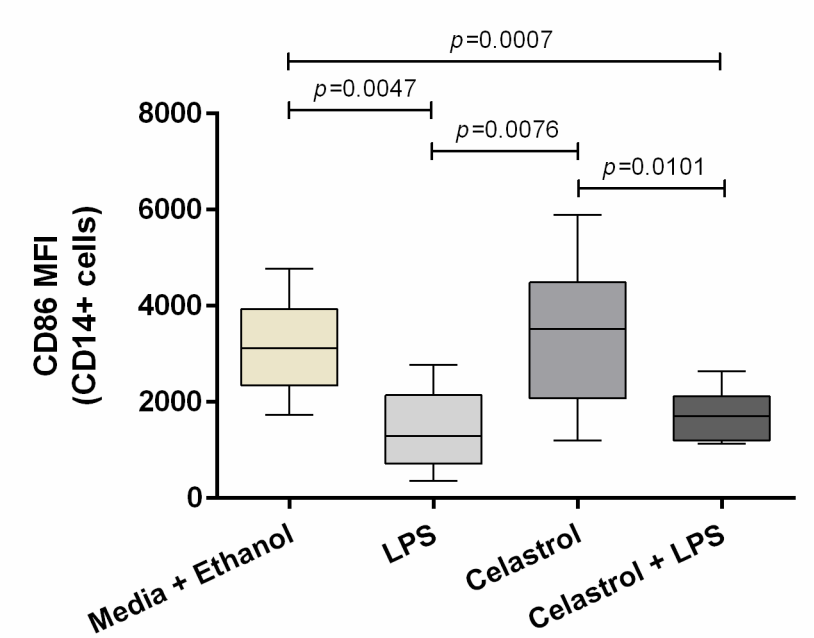

Figure 14 – Expression of CD86 (MFI) cell marker on total CD14+ cells from healthy controls is significantly reduced with LPS in comparison to Media + Ethanol, and also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol and Celastrol, and significantly increased with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

HLA-DR

Respectively to the HLA-DR expression, there were no significant differences in the levels of HLA-DR+ cells on total CD14+ cells in all tested conditions (data not shown)

Furthermore, significant differences were found in HLA-DR MFI values on total CD14+ cells.

Specifically, it was observed that Celastrol (p=0.0293) significantly reduced the expression of HLA-DR MFI cell marker on total CD14+ cells in comparison to LPS and restored the MFI values to basal levels. Celastrol + LPS significantly reduced the expression in comparison to Media + Ethanol (p=0.0155) and LPS (p=0.0047) (**Figure 15**).

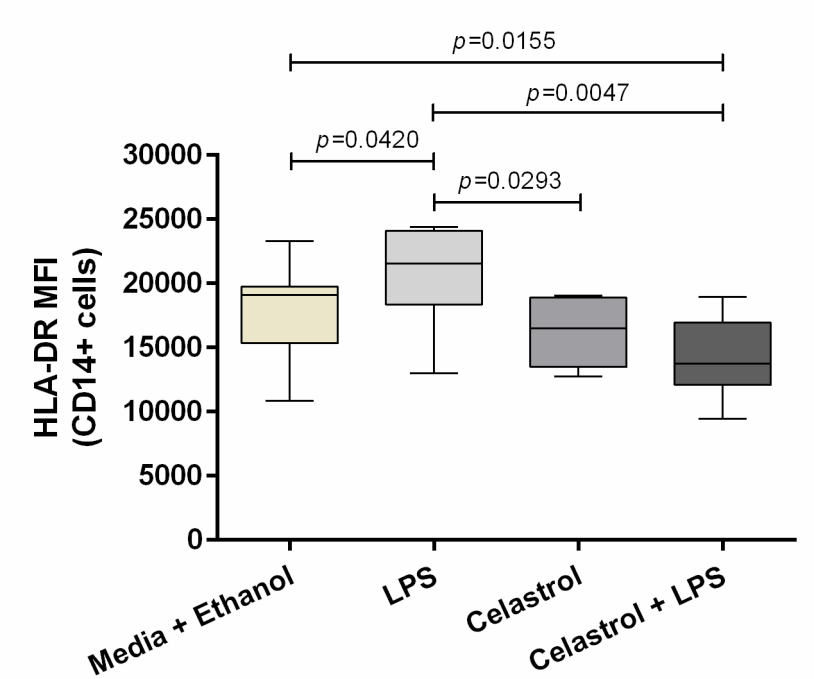

Figure 15 – Expression of HLA-DR (MFI) cell marker on total CD14+ cells from healthy controls is significantly reduced with Celastrol and Celastrol + LPS in comparison to LPS, and also significantly reduced with Celastrol + LPS in comparison to LPS, and significantly increased with LPS in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

CD115

Concerning to the expression of CD115, there were significant differences in the levels of CD115+ cells on total CD14+ cells.

Specifically, it was observed that Celastrol (p=0.0063) significantly reduced the frequencies of CD115+ cells on total CD14+ cells in comparison to Media + Ethanol, and Celastrol + LPS significantly reduced the frequencies in comparison to Media + Ethanol (p<0.0001), LPS (p=0.0110) and Celastrol (p=0.0021) (**Figure 16**).

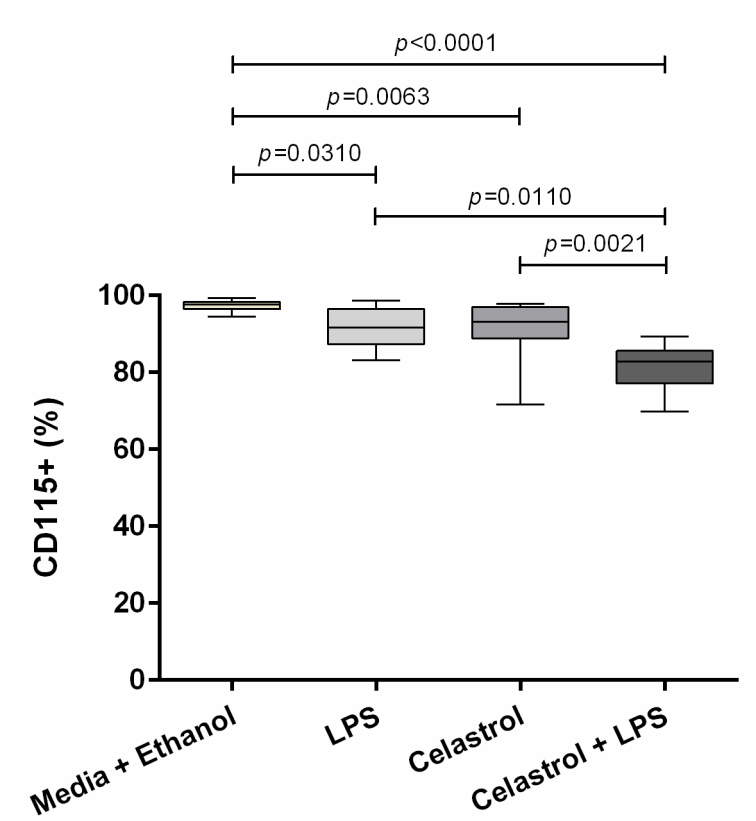

Figure 16 – Frequency of CD115+ cells on total CD14+ cells from healthy controls is significantly reduced with LPS and Celastrol conditions in comparison to Media + Ehanol, and also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocyte cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Furthermore, significant differences were found in CD115 MFI values on total CD14+ cells.

Particularly, it was observed that Celastrol (p=0.0021) significantly reduced the expression of CD115 MFI cell marker on total CD14+ cells in comparison to Media + Ethanol, and Celastrol + LPS significantly reduced the expression in comparison to Media + Ethanol (p<0.0001), LPS (p=0.0160) and Celastrol (p=0.0039) (**Figure 17**).

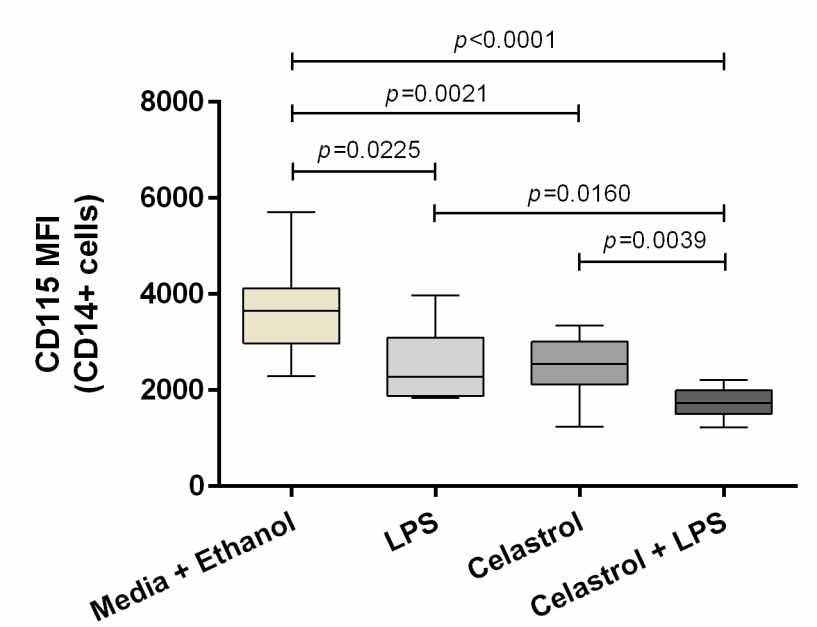

Figure 17 – Expression of CD115 (MFI) cell marker on total CD14+ cells from healthy controls is significantly reduced with LPS, Celastrol and Celastrol + LPS in comparison to Media + Ethanol, and also significantly reduced with Celastrol + LPS in comparison to LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

RANK

The analysis of RANK has revelead that no significant differences were observed in the levels of RANK+ cells and in RANK MFI values on total CD14+ cells (data not shown).

3.1.2. GRANULOCYTES

❖ *Classification of granulocyte population and the effect of celastrol*

A gate was defined around total granulocytes in peripheral leukocytes, depending upon cell size (forward scatter) and granularity (side scatter). Total granulocytes were identified based on the expression of the cell surface marker CD66b and gating out cell aggregates and dead cells through by FVD marker.

Total CD66b+ cells

The analysis of the frequency (%) of total CD66b+ cells has revealed that no statistically significant differences were found between the tested conditions in healthy controls (data not shown).

❖ *Granulocyte markers and the effect of celastrol*

The expression of several cellular markers was analyzed to characterize granulocyte phenotype upon celastrol incubation in healthy controls.

Activation and adhesion markers were used: CD11b (% and MFI) - granulocyte activation, adhesion as well as chemotaxis and phagocytosis; CD15 (% and MFI) - activation and granulocyte adhesion; CD62L (MFI), also known as L-selectin - granulocyte adhesion to inflammatory tissue. CD16 (MFI) - phagocytic potencial of granulocytes / the mediation of immunophagocytosis; CXCR2 (% and MFI), the major neutrophil/granulocyte chemoattractant receptor, also known as IL-8 receptor,beta (IL-8Rβ) - migration to inflammatory sites.

CD11b

We have observed no significant differences on the levels of CD11b+ cells on total CD66b+ cells, in all tested conditions (data not shown). However, it was observed that Celastrol (p=0.0047) significantly reduced the expression of CD11b MFI values on total

CD66b+ cells in comparison to LPS and restored the MFI values to basal levels. Celastrol + LPS significantly increased the MFI values in comparison to Media + Ethanol (p=0.0040) and Celastrol (p=0.0029) (**Figure 18**).

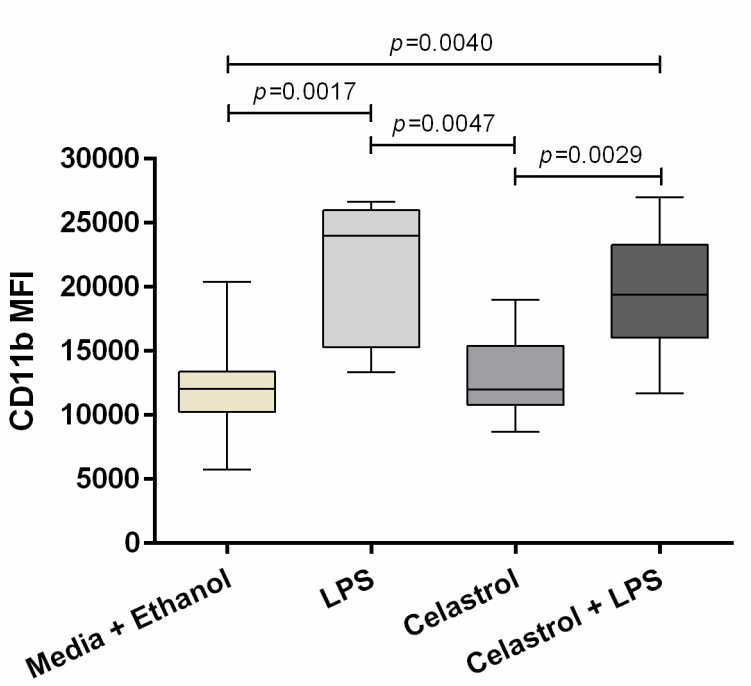

Figure 18 – Expression of CD11b (MFI) cell marker on CD66b+ cells from healthy controls is significantly increased with LPS and Celastrol + LPS conditions in comparison to Media + Ethanol, and also significantly increased with Celastrol + LPS in comparison to Celastrol, and significantly reduced with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood granulocytes cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

CD15

The analysis of CD15 has revelead that no significant differences exist in the levels of CD15+ cells and in CD15 MFI values on total CD66b+ cells (data not shown).

CD16

Respectively to the CD16 expression, there were no significant differences (data not shown).

CD62L

Regarding to the analysis of CD62L, it was observed that Celastrol (p=0.0041) and Celastrol + LPS (p=0.0089) significantly reduced the expression of CD62L MFI values on total CD66b+ cells in comparison to Media + Ethanol (**Figure 19**).

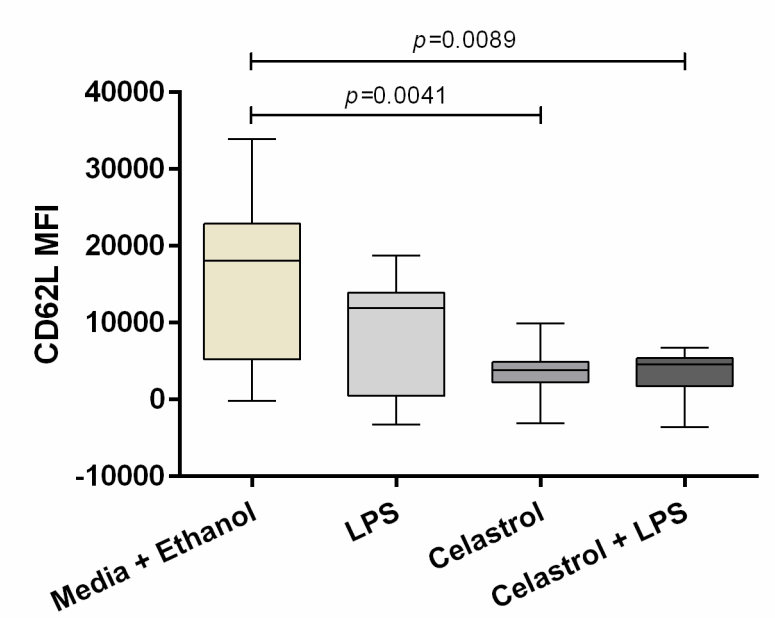

Figure 19 – Expression of CD62L (MFI) cell marker on CD66b+ cells from healthy controls is significantly reduced with Celastrol and Celastrol + LPS conditions in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood granulocytes cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

CXCR2

We have observed that Celastrol (p=0.0420) and Celastrol + LPS (p=0.0496) significantly reduced the frequencies of CXCR2+ cells on total CD66b+ cells in comparison to LPS and restored the frequencies to basal levels (**Figure 20**).

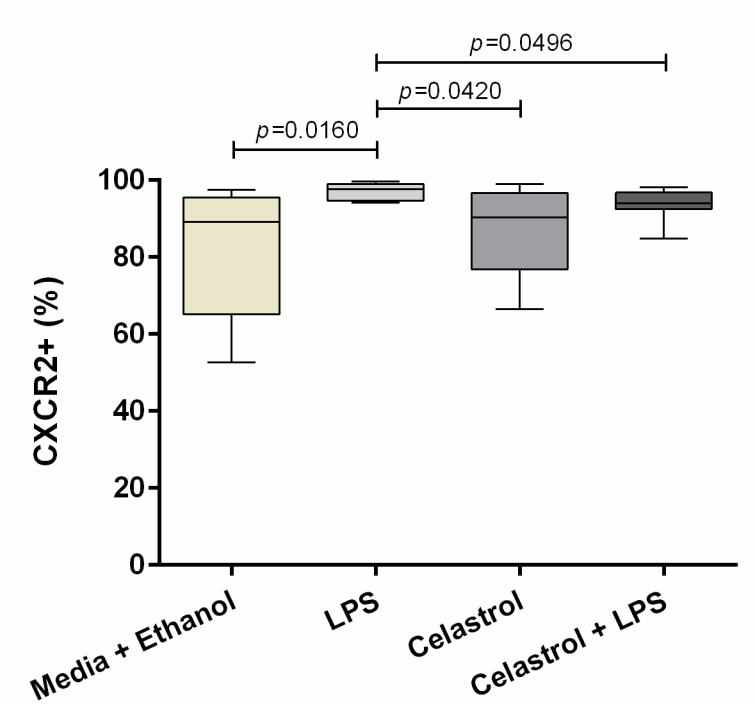

Figure 20 – Frequencies of CXCR2+ cells on total CD66b+ cells from healthy controls is significantly increased with LPS in comparison to Media + Ethanol, and significantly reduced with Celastrol and Celastrol + LPS in comparison to LPS. Data from flow cytometry analysis of peripheral blood granulocytes cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

However, no significant differences were observed in CXCR2 MFI values on total CD66b+ cells (data not shown).

3.1.3. T CELLS

❖ *Classification of T cell subpopulations and the effect of celastrol*

A gate was defined around total lymphocytes in peripheral leukocytes, depending upon cell size (forward scatter) and granularity (side scatter). Total T cells were identified based on the expression of the cell surface marker CD3 and gating out cell aggregates and dead cells through by FVD marker. T cell subpopulations were subdivided into two main subsets (gated in CD3), according to their CD4 and CD8 expression and classified as **T helper cells** (Th) (CD3+CD4+) and **T cytotoxic cells** (Tc) (CD3+CD8+).

Additionaly, **T helper cells** can be classified from **naïve T helper cells** into four major lineages: **Th1**, **Th2**, **Th17**, and **regulatory T** (**Treg**) **cells**. Each T helper cell subset exhibits characteristic functions, patterns of cytokine secretion, and expression of specific chemokine receptors.

T helper cell subsets (gated in CD3+CD4+) were classified as **naïve** (CD45RO-HLA-DR-), **naïve activated** (CD45RO-HLA-DR+), **memory** (CD45RO+HLA-DR-) and **memory activated** (CD45RO+HLA-DR+) **T cells**. To study **regulatory T** (**Treg**) **cells** (gated in CD3+CD4+), they were classified according to CD25 and Foxp3 expression (CD25+Foxp3+ subset). In addition, Treg cell analysis (gated in CD3+CD4+) was still discriminated according to the levels of CD25 expression and Treg cells classified as **CD25^{dim}** (CD4+CD25^{dim}), **CD25^{intermediate}** (CD4+CD25^{int}) and **CD25^{bright}** (CD4+CD25^{bright}) **cells**. Moreover, it was also accessed the frequency of three **T helper cell** subsets (gated in CD3+CD4+) based on the CD45RO and CCR7 expression and they were classified as **naïve** (CD45RO-CCR7+), **central memory** (CD45RO+CCR7+) and **effector memory** (CD45RO+CCR7-) **T cells**. In order to study **central memory T cells** (CD45RO+CCR7+) (gated in CD3+CD4+), they were classified as **Th1-like** (CXCR3+CCR6-), **Th2-like** (CXCR3-CCR6-) and **Th17-like** (CXCR3-CCR6+) **cells**. Likewise, in order to study **effector memory T cells** (CD45RO+CCR7-) (gated in CD3+CD4+), they were classified as **Th1-like** (CXCR3+CCR6-), **Th2-like** (CXCR3-CCR6-) and **Th17-like** (CXCR3-CCR6+) **cells**.

Concerning to the **T cytotoxic cell subsets** (gated in CD3+CD8+) they were classified as **naïve** (CD45RO-HLA-DR-), **naïve activated** (CD45RO-HLA-DR+), **memory** (CD45RO+HLA-DR-) and **memory activated** (CD45RO+HLA-DR+) **T cells**.

The frequency of T cell subsets was determined according to these classification systems, although in this thesis the graphs presented are only relative to total CD3+ T cells, due to the insufficient cell counts acquired in the gate of each subpopulation. The graphs related to T cell subpopulations are annexed as Appendix (**Appendix III**).

Total CD3+ T cells and the main T cell subpopulations (CD4+ and CD8+)

The analysis of the frequency (%) of total CD3+ T cells has revealed that no statistically significant differences were found between the tested conditions in healthy controls (data not shown). Additionally, there were no significant differences in the levels of the main T cell subpopulations (T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+)) in all conditions (data not shown).

T helper cell subpopulations (CD3+CD4+)

We have observed that Celastrol (p=0.0120) significantly reduced the levels of naïve activated T cells in comparison to LPS and restored the levels to basal levels. Celastrol + LPS significantly increased the levels in comparison to Media + Ethanol (p=0.0349) and Celastrol (p=0.0433) (**Appendix III.1**).

There were no significant differences in the levels of regulatory T (Treg) cells (CD25+Foxp3+ subset), in all tested conditions (data not shown). Additionally, no significant differences were found in the levels of CD25 subpopulations ($CD25^{dim}$, $CD25^{intermediate}$ and $CD25^{bright}$) of regulatory T (Treg) cells, in all conditions (data not shown).

Furthermore, there were no significant differences in the levels of the T helper cell subpopulations based on the CD45RO and CCR7 expression (naïve (CD45RO-CCR7+), central memory (CD45RO+CCR7+) and effector memory (CD45RO+CCR7-) T cells), in all tested conditions (data not shown).

Additionaly, no significant differences were found in the levels of central memory and effector memory T cell subpopulations, in all conditions (data not shown), except in central memory and effector memory Th2-like cells (CXCR3-CCR6-).

Specifically, it was observed that Celastrol significantly increased the levels of central

memory Th2-like cells in comparison to Media + Ethanol (p=0.0071) and LPS (p=0.0460), and Celastrol + LPS significantly increased the levels in comparison to Media + Ethanol (p=0.0001) and LPS (p=0.0030) (**Appendix III.2**). Also, it was observed that Celastrol (p=0.0195) significantly increased the levels of effector memory Th2-like cells in comparison to Media + Ethanol, and Celastrol + LPS significantly increased the levels in comparison to Media + Ethanol (p=0.0005) and LPS (p=0.0310) (**Appendix III.3**).

T cytotoxic cell subpopulations (CD3+CD8+)

Regarding to the analysis of the T cytotoxic (CD3+CD8+) cell subpopulations, no significant differences were found in the frequency of the cell subpopulations analyzed (naïve (CD45RO-HLA-DR-), naïve activated (CD45RO-HLA-DR+), memory (CD45RO+HLA-DR-) and memory activated (CD45RO+HLA-DR+) T cytotoxic cells), in all tested conditions (data not shown).

❖ *T cell markers and the effect of celastrol*

The expression of several cellular markers was analyzed to characterize T cell phenotype after incubation with celastrol in healthy controls.

Activation T cell markers were used: HLA-DR (MFI) - T cell activation; CD40L (MFI) - T cell activation and B-cell costimulation; CD25 (%) - T cell activation and particularly, the ability of Treg cells to exert suppressive or effector effects. Foxp3 (% and MFI) - suppressive effect of Treg cells (ability of Treg cells to suppress effector T-cell proliferation and cytokine secretion) and the correlation with disease severity; CD45RO (%) - memory T cells; RORgT (MFI) - differentiation of Th17 cells; CCR6 (% and MFI) - chemotaxis (migration) and recruitment of memory Th17 cells (producer of IL-17) to target tissues; CXCR3 (% and MFI) - chemotaxis (migration) and recruitment of memory Th1 cells to target tissues; CCR7 (% and MFI) - chemotaxis (migration) and recruitment of naïve or central memory Th cells to target tissues.

HLA-DR

No significant differences were found in HLA-DR MFI values on total CD3+ T cells and the main T cell subpopulations (T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+)) analyzed, in all tested conditions (data not shown).

CD40L

The analysis of the expression of CD40L has revelead that there were no significant differences in CD40L MFI values on total CD3+ T cells and the main T cell subpopulations analyzed, in all tested conditions.

RORgT

No significant differences were found in RORgT MFI values on T helper cells (CD3+CD4+), in all tested conditions (data not shown).

Foxp3

There were no significant differences in Foxp3 MFI values on regulatory T (Treg) cells (CD25+Foxp3+ subset) and on CD25 subpopulations ($CD25^{dim}$, $CD25^{intermediate}$ and $CD25^{bright}$) of regulatory T (Treg) cells, in all tested conditions (data not shown).

CCR7

No significant differences were observed in CCR7 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+), in all tested conditions (data not shown).

CCR6

There were no significant differences in CCR6 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+), in all tested conditions (data not shown).

CXCR3

We have observed that Celastrol (p=0.0107) significantly reduced the expression of CXCR3 MFI values on total CD3+ T cells in comparison to Media + Ethanol, and Celastrol + LPS significantly reduced the expression in comparison to Media + Ethanol (p=0.0002), LPS (p=0.0029) and Celastrol (p=0.0197) (**Figure 21**).

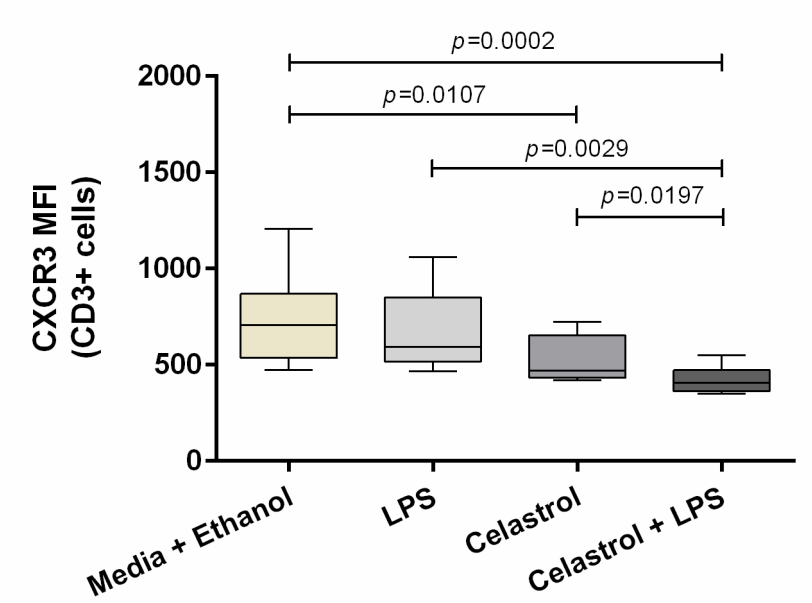

Figure 21 – Expression of CXCR3 (MFI) cell marker on total CD3+ T cells from healthy controls is significantly reduced with Celastrol in comparison to Media + Ethanol, and is also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood T cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Also, it was observed that Celastrol + LPS significantly reduced the expression of CXCR3 MFI values on T helper cells (CD3+CD4+) in comparison to Media + Ethanol (p=0.0368) and LPS (p=0.0312) (**Appendix III.4**).

3.1.4. B CELLS

❖ *Classification of B cell subpopulations and the effect of celastrol*

A gate was defined around total lymphocytes in peripheral leukocytes, depending upon cell size (forward scatter) and granularity (side scatter). Total B cells were identified based on the expression of the cell surface marker CD19, gating out cell aggregates and dead cells through by FVD marker, and its subpopulations were classified using three classification systems (gated in CD19): according to IgD and CD27 expression - **naïve B cells** (IgD+CD27-), **pre-switch memory** (IgD+CD27+), **post-switch memory** (IgD-CD27+) and **double-negative** (DN) (IgD-CD27-) **B cells** (Moura RA et al., 2017); according to IgD and CD38 expression – **transitional** (IgD+CD38++) **B cells** and **plasmablasts** (IgD-CD38++) (Moura RA et al., 2017); and according to CD21 and CD38 expression – **activated B cells** ($CD21^{low}CD38^{low}$) (Wehr C et al., 2008).

Although an analysis with these classification systems has been performed in the present work to define B cell subpopulations, the graphs with significantly results presented in this thesis are only relative to total CD19+ B cells, while that graphs related to its subpopulations are annexed as Appendix (**Appendix IV**). This is due to the low number of cell counts obtained in some samples during data acquisition, which limited the analysis robustness of several B cell subpopulations.

Total CD19+ B cells and B cell subpopulations

The analysis of the frequency (%) of total CD19+ B cells has revealed that no statistically significant differences were found between the tested conditions in healthy controls (data not shown). Additionally, there were no significant differences in the levels of the B cell subpopulations (naïve, pre-switch memory, post-switch memory, double-negative B cells and transitional B cells, plasmablasts – according to the IgD/CD27 and IgD/CD38 classification system, respectively) in all conditions (data not shown).

❖ *B cell markers and the effect of celastrol*

The expression of several cellular markers was analyzed to characterize B cell phenotype upon celastrol incubation for 4h in healthy controls.

B cell markers were used according to their function: BAFF-R (% and MFI) - activation and survival; HLA-DR (% and MFI) - activation and its function as antigen-presentation cell (APC) as well as T-cell costimulation; CD21 (% and MFI) - immature B cells and complement system activation ($CD21^{low}$, autoimmunity); CD38 (MFI) - adhesion, activation, and calcium signaling; CD32 or FcgRIIB (MFI) - inhibition; RANKL (% and MFI) - osteoclast activation, differentiation and survival (osteoclastogenesis) and bone resorption; CD95 (% and MFI), also known as Fas receptor (FasR) - Fas-mediated apoptosis.

BAFF-R

We have observed that no significant differences exist in the frequency of BAFF-R+ cells and in BAFF-R MFI values on total CD19+ B cells and in all B cell subpopulations, in all tested conditions (data not shown).

FcgRIIB

Regarding FcgRIIB, no significant differences were found in FcgRIIB MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown), except in plasmablasts (IgD-CD38++). We observed that Celastrol (p=0.0047) and Celastrol + LPS (p=0.0176) significantly reduced the expression of FcgRIIB MFI cell marker in peripheral plasmablasts in comparison to LPS and restored the MFI values to basal levels (**Appendix IV.1**).

CD38

The analysis of CD38 has revelead that no significant differences were observed in CD38 MFI values on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions (data not shown).

CD21

Respectively to the CD21 expression, there were no significant differences in the levels of CD21+ cells on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions (data not shown).

Moreover, it was observed that Celastrol (p=0.0415) significantly increased the expression of CD21 MFI cell marker on total CD19+ B cells, in comparison to LPS and restored the MFI values to basal levels (**Figure 22**). However, no significant differences were found in CD21 MFI values on the remaining B cell subpopulations analyzed, in all tested conditions (data not shown), except in naïve B cells (IgD+CD27-). Herein, it was observed that Celastrol (p=0.0490) significantly increased the expression of CD21 MFI cell marker in peripheral naïve B cells in comparison to LPS (**Appendix IV.2**).

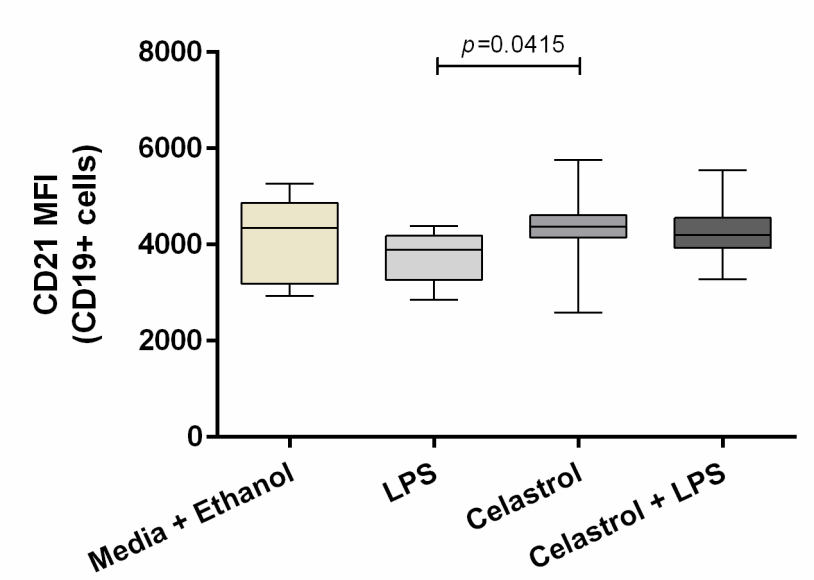

Figure 22 – Expression of CD21 (MFI) cell marker on total CD19+ B cells from healthy controls is significantly increased with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

$CD21^{low}CD38^{low}$

Concerning to the expression of $CD21^{low}CD38^{low}$, there were no significant differences in the levels of $CD21^{low}CD38^{low}$+ cells on total CD19+ B cells, in all tested conditions (data not shown).

HLA-DR

The analysis of HLA-DR has revelead that no significant differences were observed in the levels of HLA-DR+ cells on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions (data not shown), except in transitional B cells (IgD+CD38++). Herein, it was observed that Celastrol + LPS (p=0.0230) significantly increased the frequencies of HLA-DR+ cells on transitional B cells in comparison to LPS and restored the frequencies to basal levels (**Appendix IV.3**). Furthermore, no significant differences were found in HLA-DR MFI values on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions (data not shown).

RANKL

Regarding to the analysis of RANKL, there were significant differences in the levels of RANKL+ cells on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions, except in double-negative B cells (IgD-CD27-) and plasmablasts (IgD-CD38++) (data not shown).

Specifically, it was observed that Celastrol (p=0.0278) significantly reduced the frequencies of RANKL+ cells on total CD19+ B cells in comparison to LPS and restored the frequencies to basal levels (**Figure 23**).

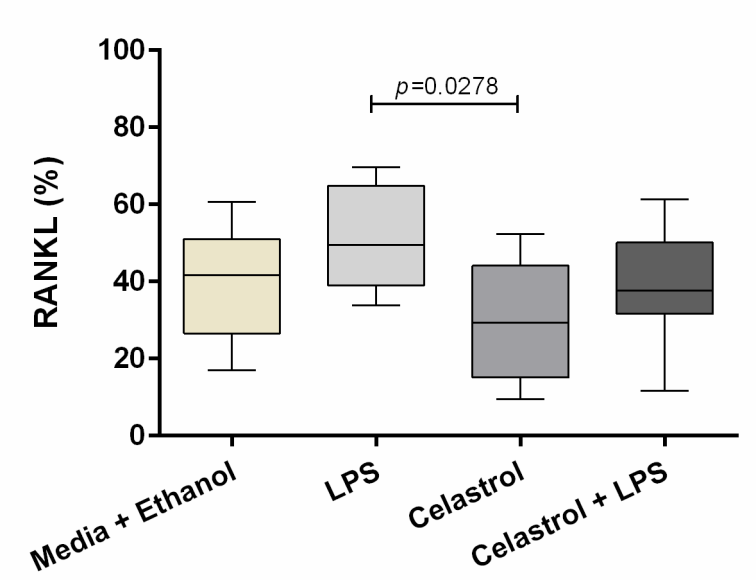

Figure 23 – Frequency of RANKL+ cells on total CD19+ B cells from healthy controls is significantly reduced with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Similarly, it was observed that Celastrol significantly reduced the frequencies of RANKL+ cells on naïve B cells (IgD+CD27-) (p=0.0420) (**Appendix IV.4**), pre-switch memory (IgD+CD27+) (p=0.0140) (**Appendix IV.5**) and on post-switch memory (IgD-CD27+) (p=0.0177) (**Appendix IV.6**) in comparison to LPS and restored these frequencies to basal levels. And lastly, it was observed that Celastrol significantly reduced the frequencies of RANKL+ cells on transitional B cells (IgD+CD38++) in comparison to Media + Ethanol (p=0.0371) and LPS (p=0.0062) (**Appendix IV.7**).

Furthermore, no significant differences were found in RANKL MFI values on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions (data not shown).

CD95

Respectively to the CD95 expression, there were no significant differences in the levels of CD95+ cells on total CD19+ B cells and all B cell subpopulations analyzed, in all tested conditions (data not shown), except in naïve B cells, transitional B cells and plasmablasts.

Specifically, it was observed that Celastrol (p=0.0175) significantly reduced the frequencies of CD95+ cells on naïve B cells (IgD+CD27-) in comparison to LPS and restored the frequencies to basal levels (**Appendix IV.8**). Similarly, it was observed that Celastrol significantly reduced the frequencies of CD95+ cells on transitional B cells (IgD+CD38++) in comparison to Media + Ethanol (p=0.0220) and LPS (p=0.0040) (**Appendix IV.9**). Also, it was observed that Celastrol (p=0.0190) significantly reduced the frequencies of CD95+ cells on plasmablasts (IgD-CD38++) in comparison to LPS and restored the frequencies to basal levels (**Appendix IV.10**).

Furthermore, no significant differences were found in CD95 MFI values (data not shown), except in naïve B cells, transitional B cells and plasmablasts.

We have observed that Celastrol (p=0.0155) significantly reduced the expression of CD95 MFI values on naïve B cells (IgD+CD27-) in comparison to Media + Ethanol (**Appendix IV.11**). In addition, it was observed that Celastrol (p=0.0068) and Celastrol + LPS (p=0.0349) significantly reduced the expression of CD95 MFI values on transitional B cells (IgD+CD38++) in comparison to Media + Ethanol (**Appendix IV.12**). Also, it was observed that Celastrol (p=0.0190) significantly reduced the expression of CD95 MFI values on plasmablasts (IgD-CD38++) in comparison to LPS and restored the MFI values to basal levels (**Appendix IV.13**).

3.2. CHRONIC RA PATIENTS

3.2.1. MONOCYTES

❖ *Effect of celastrol on monocyte population*

Total CD14+ cells

The analysis of the frequency of total CD14+ cells has revealed that no statistically significant differences were found between the tested conditions in chronic RA patients (data not shown).

❖ *Effect of celastrol on monocyte markers*

CD16

Regarding CD16, no significant differences were observed in the levels of CD16+ cells and in CD16 MFI values on total CD14+ cells in all tested conditions (data not shown).

CD86

The analysis of the expression of CD86 has revelead that Celastrol + LPS (p=0.0159) significantly reduce the frequencies of CD86+ cells on total CD14+ cells in comparison to Media + Ethanol (**Figure 24**).

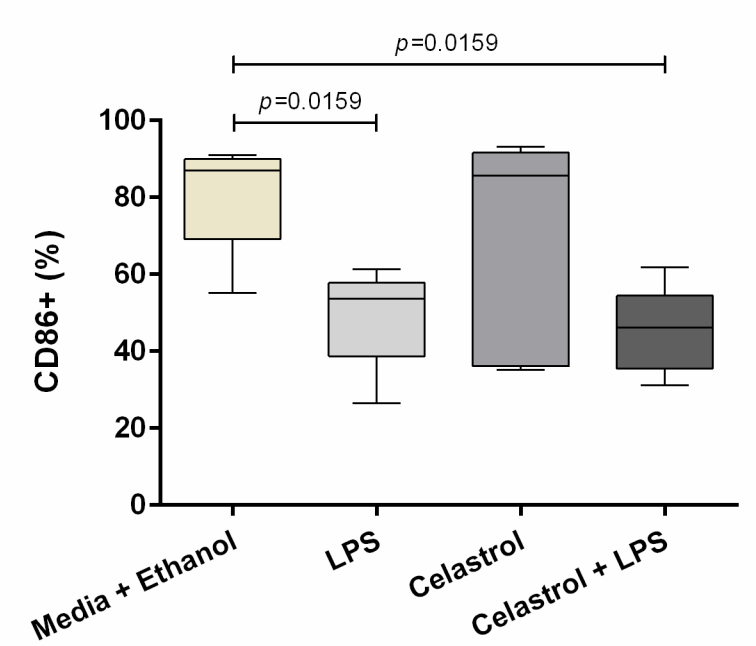

Figure 24 – Frequency of CD86+ cells on total CD14+ cells from chronic RA patients is significantly reduced with LPS and Celastrol + LPS in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood monocyte cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Furthermore, no significant differences were found in CD86 MFI values on total CD14+ cells (data not shown).

HLA-DR

Respectively to the HLA-DR expression, there were no significant differences in the levels of HLA-DR+ cells and in HLA-DR MFI values on total CD14+ cells (data not shown).

CD115

Concerning to the expression of CD115, it was observed that Celastrol (p=0.0079) and Celastrol + LPS (p=0.0079) significantly reduced the frequencies of CD115+ cells on total CD14+ cells in comparison to Media + Ethanol (**Figure 25**).

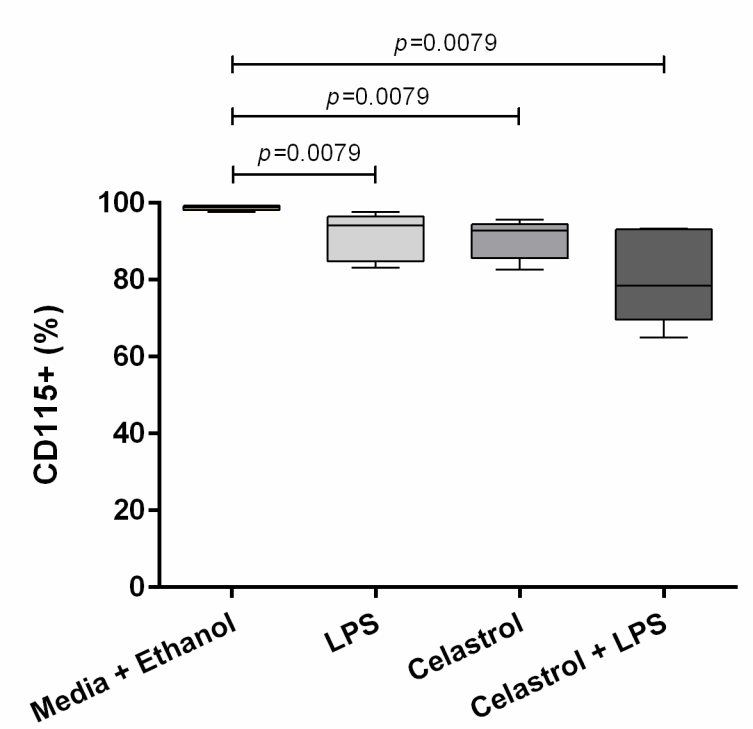

Figure 25 – Frequency of CD115+ cells on total CD14+ cells from chronic RA patients is significantly reduced with LPS, Celastrol and Celastrol + LPS conditions in comparison to Media + Ehanol. Data from flow cytometry analysis of peripheral blood monocyte cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Furthermore, no significant differences were found in CD115 MFI values on total CD14+ cells (data not shown).

RANK

The analysis of the expression of RANK has revelead that no significant differences were observed in the levels of RANK+ cells and in RANK MFI values on total CD14+ cells (data not shown).

3.2.2. GRANULOCYTES

❖ *Effect of celastrol on granulocyte population*

Total CD66b+ cells

The analysis of the frequency of total CD66b+ cells has revealed that no statistically significant differences were found between the tested conditions in chronic RA patients (data not shown).

❖ *Effect of celastrol on granulocyte markers*

CD11b

Regarding to the analysis of CD11b, no significant differences were observed in its levels on total CD66b+ cells (data not shown). However, it was observed that Celastrol (p=0.0047) significantly reduced the expression of CD11b MFI values on total CD66b+ cells in comparison to LPS and restored the MFI values to basal levels (**Figure 26**).

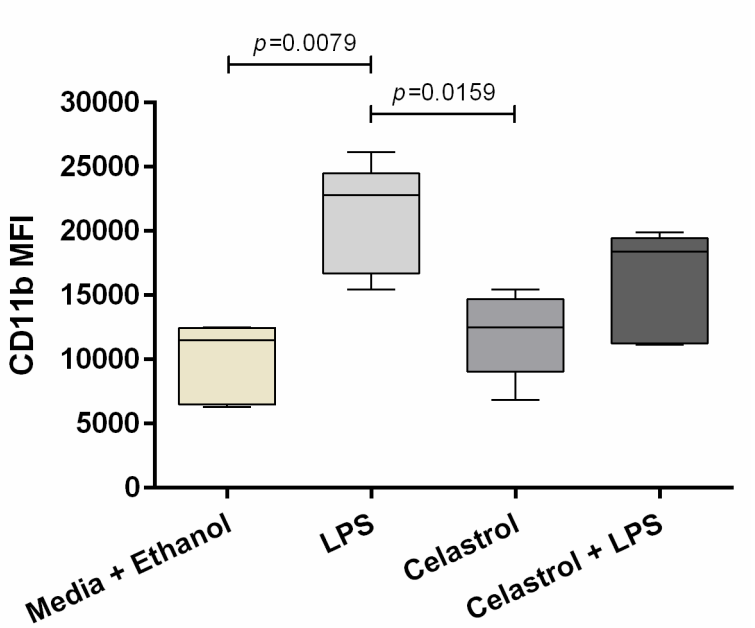

Figure 26 – Expression of CD11b (MFI) cell marker on CD66b+ cells from chronic RA patients is significantly increased with LPS in comparison to Media + Ethanol and significantly reduced with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood granulocytes cells from chronic RA patients tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

CD15

The analysis of the expression of CD15 has revelead that no significant differences were observed in the levels of CD15+ cells and in CD15 MFI values on total CD66b+ cells (data not shown).

CD16

Respectively to the CD16 expression, there were no significant differences (data not shown).

CD62L

Regarding to the analysis of CD62L, there were no significant differences in the CD62L MFI values on total CD66b+ cells (data not shown).

CXCR2

Respectively to the CXCR2 expression, there were no significant differences in the levels of CXCR2+ cells and in CXCR2 MFI values on total CD66b+ cells (data not shown).

3.2.3. T CELLS

❖ *Effect of celastrol on T cell subpopulations*

Total CD3+ T cells and the main T cell subpopulations (CD4+ and CD8+)

The analysis of the frequency of total CD3+ T cells has revealed that no statistically significant differences were found between the tested conditions in chronic RA patients (data not shown). Additionally, there were no significant differences in the levels of the main T cell subpopulations (T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+)) in all conditions (data not shown).

T helper cell subpopulations (CD3+CD4+)

We have observed that Celastrol (p=0.0317) significantly increased the levels of memory activated T cells in comparison to Media + Ethanol (**Appendix III.5**).

There were no significant differences in the levels of regulatory T (Treg) cells (CD25+Foxp3+ subset) (data not shown). Additionally, no significant differences were found in the levels of CD25 subpopulations ($CD25^{dim}$, $CD25^{intermediate}$ and $CD25^{bright}$) of regulatory T (Treg) cells (data not shown).

Furthermore, there were no significant differences in the levels of the T helper cell subpopulations based on the CD45RO and CCR7 expression (naïve (CD45RO-CCR7+), central memory (CD45RO+CCR7+) and effector memory (CD45RO+CCR7-) T cells) (data not shown).

Additionaly, no significant differences were found in the levels of central memory and effector memory T cell subpopulations (Th1-like (CXCR3+CCR6-), Th2-like (CXCR3-CCR6-) and Th17-like (CXCR3-CCR6+) cells) (data not shown).

T cytotoxic cell subpopulations (CD3+CD8+)

Regarding to the analysis of the T cytotoxic (CD3+CD8+) cell subpopulations, no significant differences were found in the frequency of the cell subpopulations analyzed (naïve (CD45RO-HLA-DR-), naïve activated (CD45RO-HLA-DR+), memory (CD45RO+HLA-DR-) and memory activated (CD45RO+HLA-DR+) T cytotoxic cells) (data not shown).

- *Effect of celastrol on T cell markers*

HLA-DR

Regarding to the analysis of HLA-DR, no significant differences were found in HLA-DR MFI values on total CD3+ T cells and the main T cell subpopulations (T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+)) analyzed (data not shown). CORRECTO

CD40L

The analysis of the expression of CD40L has revelead that no significant differences were observed in CD40L MFI values on total CD3+ T cells and the main T cell subpopulations analyzed.

RORgT

Respectively to the RORgT expression, there were no significant differences in RORgT MFI values on T helper cells (CD3+CD4+) (data not shown).

Foxp3

Concerning to the expression of Foxp3, there were no significant differences in Foxp3 MFI values on regulatory T (Treg) cells (CD25+Foxp3+ subset) and on CD25 subpopulations ($CD25^{dim}$, $CD25^{intermediate}$ and $CD25^{bright}$) of regulatory T (Treg) cells (data not shown).

CCR7

The analysis of CCR7 has revelead that no significant differences were observed in CCR7 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+) (data not shown).

CCR6

Regarding to the analysis of CCR6, there were no significant differences in CCR6 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+) (data not shown).

CXCR3

Respectively to the CXCR3 expression, there were no significant differences in CXCR3 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+) (data not shown).

3.2.4. B CELLS

❖ *Effect of celastrol on B cell subpopulations*

Total CD19+ B cells and B cell subpopulations

The analysis of the frequency of total CD19+ B cells has revealed that no statistically significant differences were found between the tested conditions in chronic RA patients (data not shown). Additionally, there were no significant differences in the levels of the B cell subpopulations (naïve, pre-switch memory, post-switch memory, double-negative B cells and transitional B cells, plasmablasts) in all conditions (data not shown).

❖ *Effect of celastrol on B cell markers*

BAFF-R

We have observed that no significant differences exist in the frequency of BAFF-R+ cells and in BAFF-R MFI values on total CD19+ B cells and in all B cell subpopulations, in all tested conditions (data not shown).

FcgRIIB

Regarding to the analysis of FcgRIIB, no significant differences were found in FcgRIIB MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown).

CD38

The analysis of the expression of CD38 has revelead that no significant differences were observed in CD38 MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown).

CD21

Respectively to the CD21 expression, there were no significant differences in the levels of CD21+ cells and in CD21 MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown).

$CD21^{low}CD38^{low}$

Concerning to the expression of $CD21^{low}CD38^{low}$, there were no significant differences in the levels of $CD21^{low}CD38^{low}$+ cells on total CD19+ B cells (data not shown).

HLA-DR

The analysis of HLA-DR has revelead that no significant differences were observed in the levels of HLA-DR+ cells and in HLA-DR MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown).

RANKL

Regarding to the analysis of RANKL, there were no significant differences in the levels of RANKL+ cells and in RANKL MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown).

CD95

Respectively to the CD95 expression, there were no significant differences in the levels of CD95+ cells and in CD95 MFI values on total CD19+ B cells and all B cell subpopulations analyzed (data not shown).

3.3. CHRONIC RA PATIENTS vs HEALTHY CONTROLS

3.3.1. MONOCYTES

❖ *Effect of celastrol on monocyte population*

Total CD14+ cells

The analysis of the frequency of total CD14+ cells has revealed that statistically significant differences were found, when comparing chronic RA patients with healthy controls.

Specifically, it was observed that leukocytes from chronic RA patients tested with LPS (p=0.0173) condition had a significantly increased frequency of total CD14+ cells, and when tested with Celastrol (p=0.0290), had a significantly reduced frequency in comparison to healthy controls (**Figure 27**). A linear downward trend is observed in Media + Ethanol, Celastrol and Celastrol + LPS conditions, which leads us to assume that the significant difference observed in Celastrol is due to the disease phenotype and not to the effect of the compound. However, the significant difference observed in LPS condition seems to indicate that the LPS addition reduces the frequency of total CD14+ cells in healthy controls.

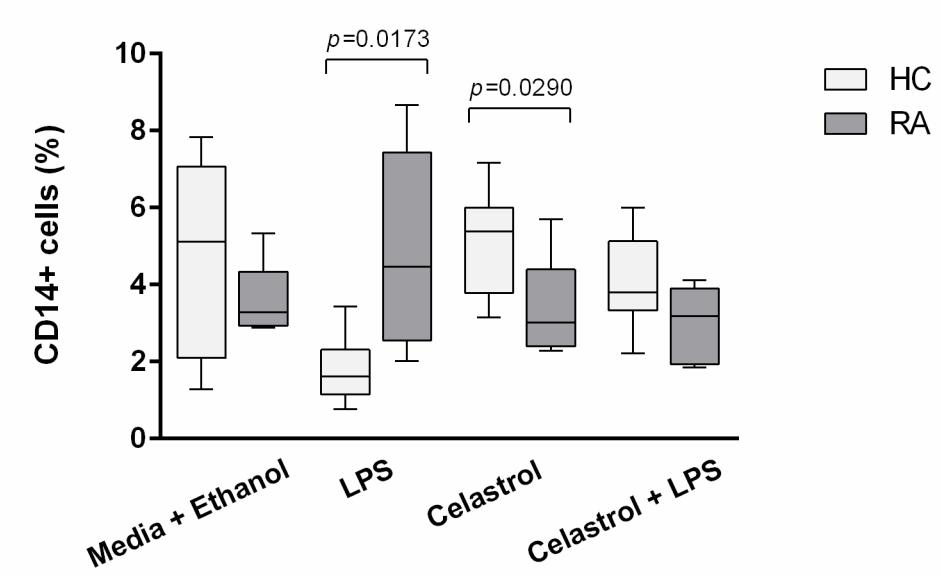

Figure 27 – Frequency of CD14+ cells in chronic RA patients tested with LPS is significantly increased in comparison to healthy controls, and tested with Celastrol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

❖ *Effect of celastrol on monocyte markers*

CD16

Regarding to CD16, it was observed that no significant differences were observed in the levels of CD16+ cells and in CD16 MFI values on total CD14+ cells, when comparing chronic RA patients with healthy controls (data not shown).

CD86

The analysis of CD86 has revelead that significant differences were observed in the levels of CD86+ cells and in CD86 MFI values on total CD14+ cells, when comparing chronic RA patients with healthy controls.

Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0007), Celastrol (p=0.0185) and Celastrol + LPS (p=0.0007) conditions, had significantly reduced frequencies of CD86+ cells on total CD14+ cells in comparison to healthy controls (**Figure 28**).

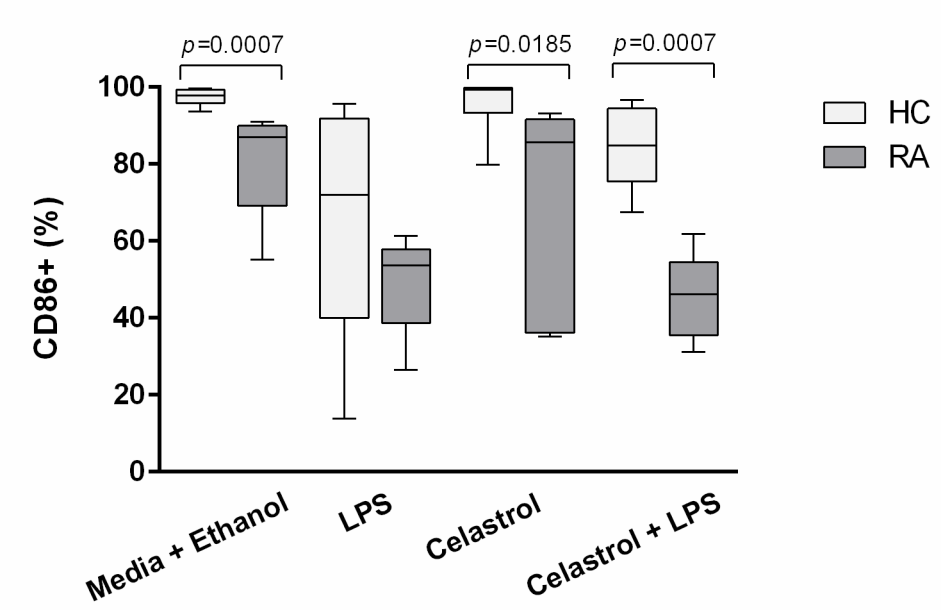

Figure 28 – Frequency of CD86+ cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Also, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0007), Celastrol (p=0.0070) and Celastrol + LPS (p=0.0007) conditions, had significantly reduced expression of CD86 MFI values on total CD14+ cells in comparison to healthy controls (**Figure 29**).

Both in the levels of CD86+ cells and in CD86 MFI values on total CD14+ cells, a linear downward trend is observed in Media + Ethanol and Celastrol conditions, which leads us to assume that the significant differences observed in these conditions are due to the disease phenotype and not to the effect of the compound. However, the significant difference observed in Celastrol + LPS condition seems to indicate that the LPS addition reduces the levels of CD86+ cells on total CD14+ cells in chronic RA patients and reduces the CD86 MFI values on total CD14+ cells in healthy controls.

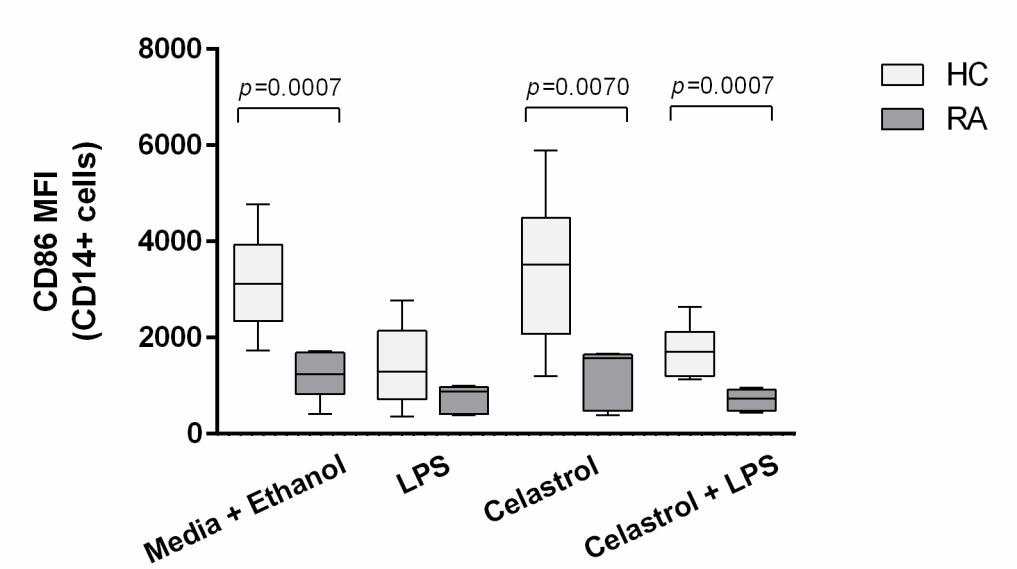

Figure 29 – Expression of CD86 (MFI) cell marker on CD14+ cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

HLA-DR

Respectively to the HLA-DR expression, there were no significant differences in the levels of HLA-DR+ cells on total CD14+ cells, when comparing chronic RA patients with healthy controls (data not shown).

However, significant differences were found in HLA-DR MFI values on total CD14+ cells, when comparing chronic RA patients with healthy controls. It was observed that

leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0047), Celastrol (p=0.0016) and Celastrol + LPS (p=0.0127) conditions, had significantly reduced expression of HLA-DR MFI values on total CD14+ cells in comparison to healthy controls (**Figure 30**). A linear downward trend is observed in Media + Ethanol, Celastrol and Celastrol + LPS conditions, which leads us to assume that the significant differences observed in these conditions are due to the disease phenotype and not to the effect of the added stimuli. However, the LPS condition seems to indicate that the LPS addition increases the HLA-DR MFI values on total CD14+ cells both in healthy controls and in chronic RA patients.

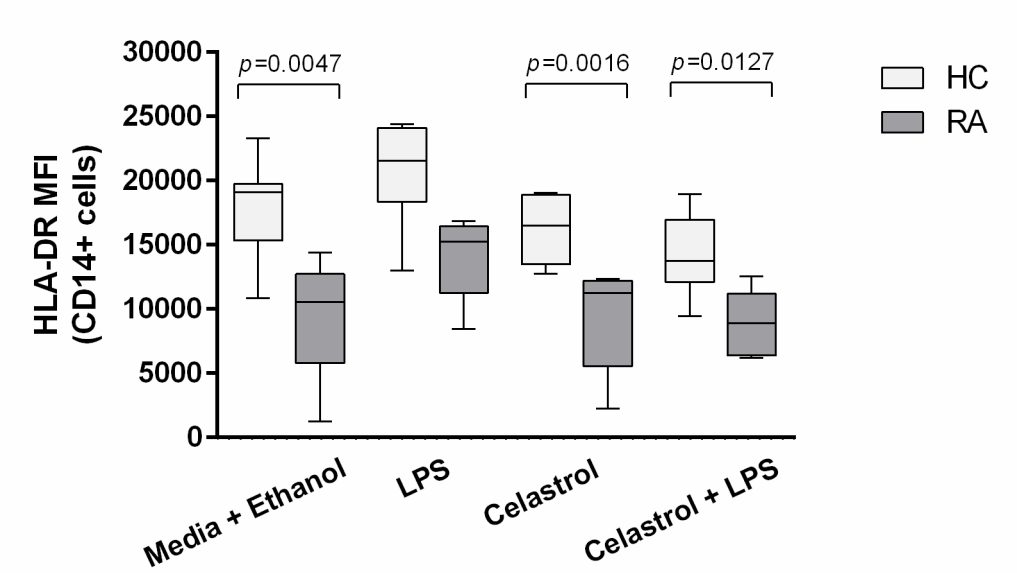

Figure 30 – Expression of HLA-DR (MFI) cell marker on CD14+ cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

CD115

Concerning to the expression of CD115, no significant differences were found in the levels of CD115+ cells and in CD115 MFI values on total CD14+ cells, when comparing chronic RA patients with healthy controls (data not shown).

RANK

The analysis of the expression of RANK has revelead that no significant differences were observed in the levels of RANK+ cells and in RANK MFI values on total CD14+ cells, when comparing chronic RA patients with healthy controls (data not shown).

3.3.2. GRANULOCYTES

❖ *Effect of celastrol on granulocyte population*

Total CD66b+ cells

The analysis of the frequency of total CD66b+ cells has revealed that leukocytes from chronic RA patients tested with Celastrol (p=0.0133) and Celastrol + LPS (p=0.0376) conditions, had significantly increased frequencies of total CD66b+ cells in comparison to healthy controls (**Figure 31**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

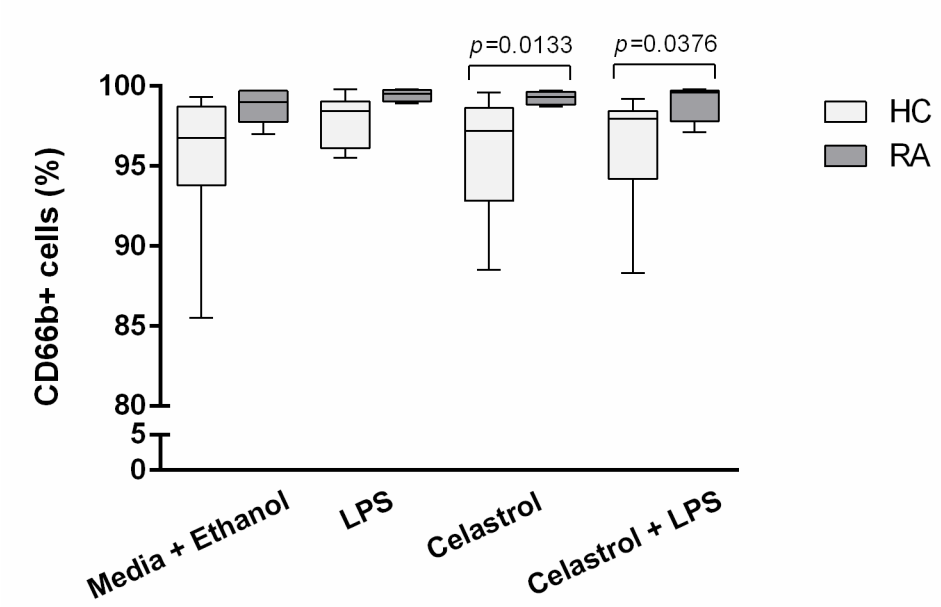

Figure 31 – Frequency of total CD66b+ cells in chronic RA patients tested with Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

❖ *Effect of celastrol on granulocyte markers*

CD11b

Regarding to the analysis of CD11b, no significant differences were observed in the levels of CD11b+ cells and in CD11b MFI values on total CD66b+ cells, when comparing chronic RA patients with healthy controls (data not shown).

CD15

The analysis of the expression of CD15 has revelead that leukocytes from chronic RA patients tested with Celastrol (p=0.0043) condition, had significantly increased frequencies of CD15+ cells on total CD66b+ cells in comparison to healthy controls (**Figure 32**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

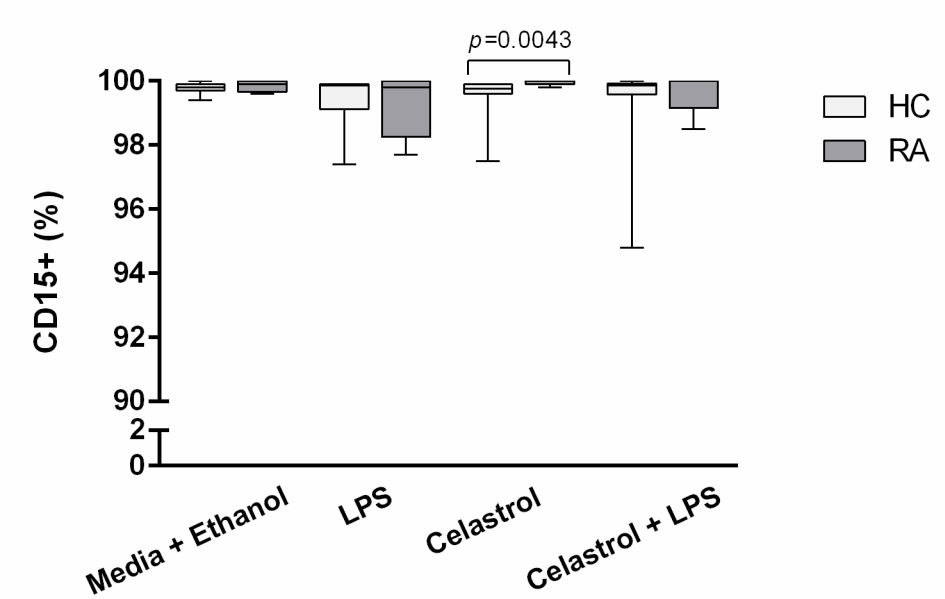

Figure 32 – Frequency of CD15+ cells on total CD66b+ cells in chronic RA patients tested with Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Furthermore, no significant differences were found in CD15 MFI values on total CD66b+ cells, when comparing chronic RA patients with healthy controls (data not shown).

CD16

Respectively to the CD16 expression, there were significant differences in the levels of $CD16^{high}$ cells and $CD16^{low}$ cells on total CD66b+ cells, when comparing chronic RA patients with healthy controls.

Specifically, it was revelead that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0420) and Celastrol + LPS (p=0.0290) conditions, had significantly increased frequencies of $CD16^{high}$ cells on total CD66b+ cells in comparison to healthy controls (**Figure 33**).

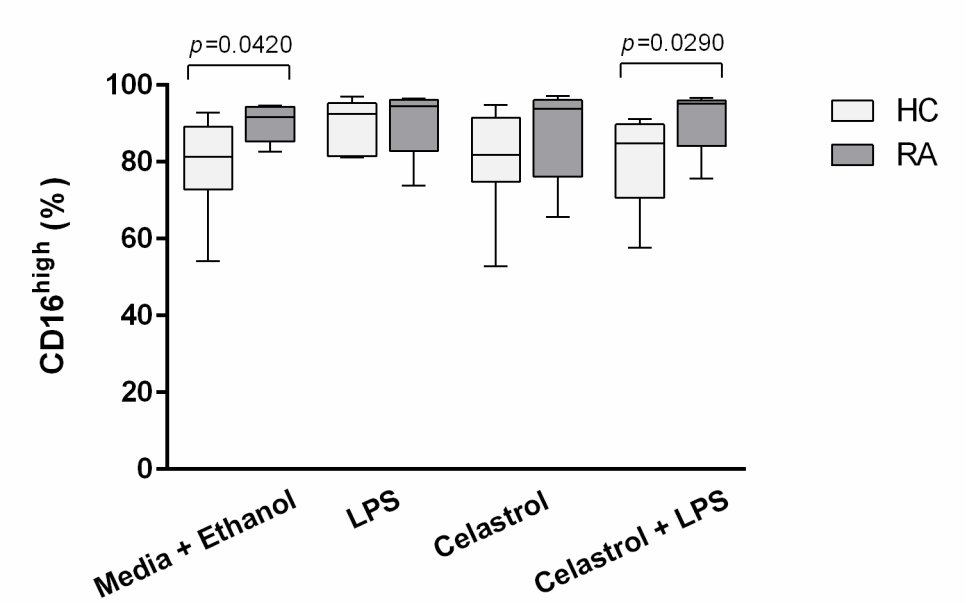

Figure 33 – Frequency of $CD16^{high}$ cells on total CD66b+ cells in chronic RA patients tested with Media + Ethanol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

In addition, with Celastrol + LPS (p=0.0080) condition, it was observed a significantly reduced frequency of $CD16^{low}$ cells on total CD66b+ cells in comparison to healthy controls (**Figure 34**).

Both in the levels of $CD16^{high}$ cells and $CD16^{low}$ cells on total CD66b+ cells, a linear downward and increasing trend, respectively, is observed in Media + Ethanol, Celastrol and Celastrol + LPS conditions, which leads us to assume that the significant difference observed in Celastrol + LPS condition is due to the disease phenotype and not to the effect of the added stimuli. However, the LPS condition seems to indicate that the LPS addition

increases the levels of $CD16^{high}$ cells on total CD66b+ cells and reduces the levels of $CD16^{low}$ cells on total CD66b+ cells in healthy controls.

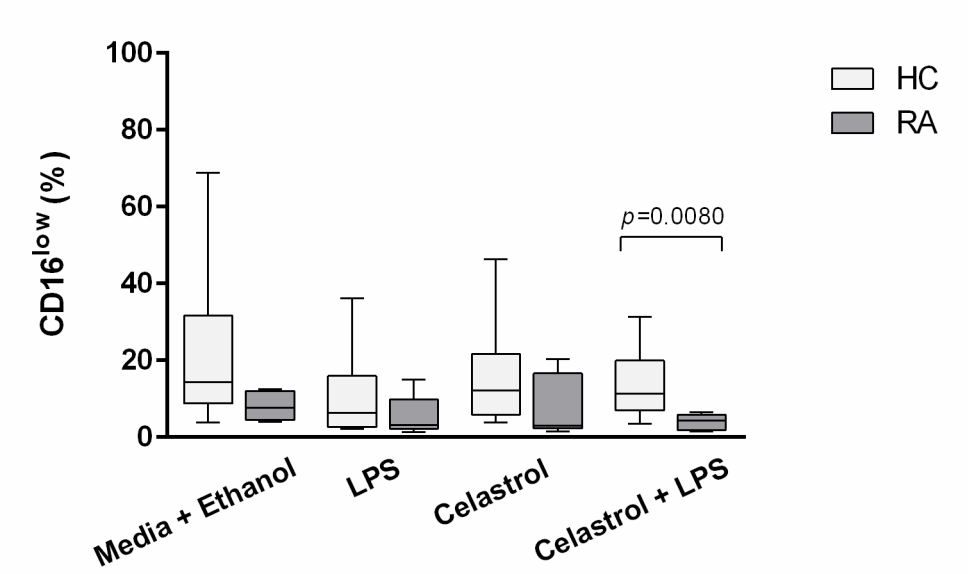

Figure 34 – Frequency of $CD16^{low}$ cells on total CD66b+ cells in chronic RA patients tested with Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

However, no significant differences were found in $CD16^{high}$ MFI values, $CD16^{low}$ MFI values and CD16 MFI values on total CD66b+ cells, when comparing chronic RA patients with healthy controls (data not shown).

CD62L

Regarding to the analysis of CD62L, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0013), Celastrol (p=0.0120) and Celastrol + LPS (p=0.0027) conditions, had significantly reduced expression of CD62L MFI values on total CD66b+ cells in comparison to healthy controls (**Figure 35**). The levels of CD62L cells are reduced in chronic RA patients, and as they are pre-primed they respond more when addeding LPS, then no significant difference was observed in this condition.

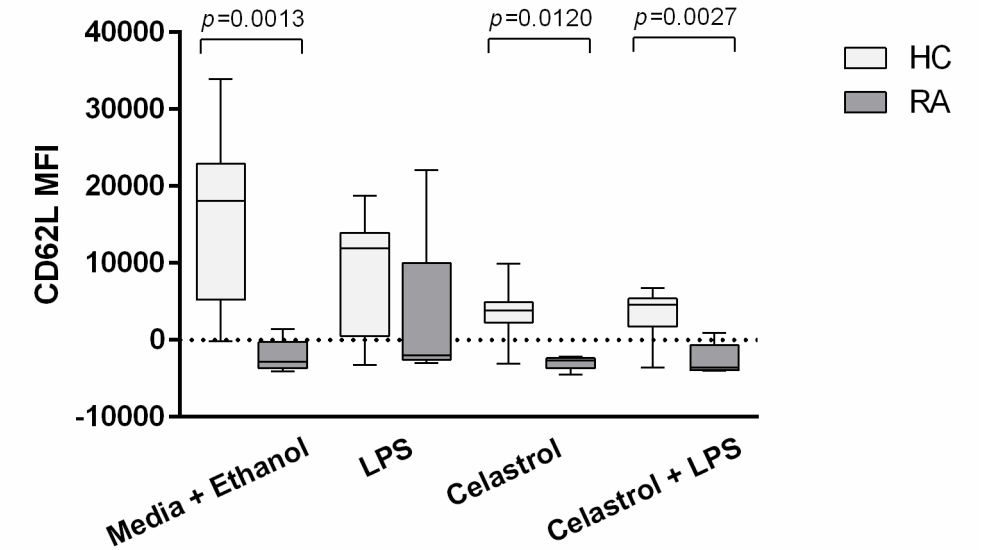

Figure 35 – Expression of CD62L (MFI) cell marker on CD66b+ cells on chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

CXCR2

Respectively to the CXCR2 expression, there were significant differences in the levels of CXCR2+ cells and in CXCR2 MFI values on total CD66b+ cells, when comparing chronic RA patients with healthy controls (data not shown). CORRECTO

Specifically, it was observed that leukocytes from chronic RA patients tested with Celastrol + LPS (*p*=0.0115) condition, had a significantly increased frequency of CXCR2+ cells on total CD66b+ cells in comparison to healthy controls (**Figure 36**).

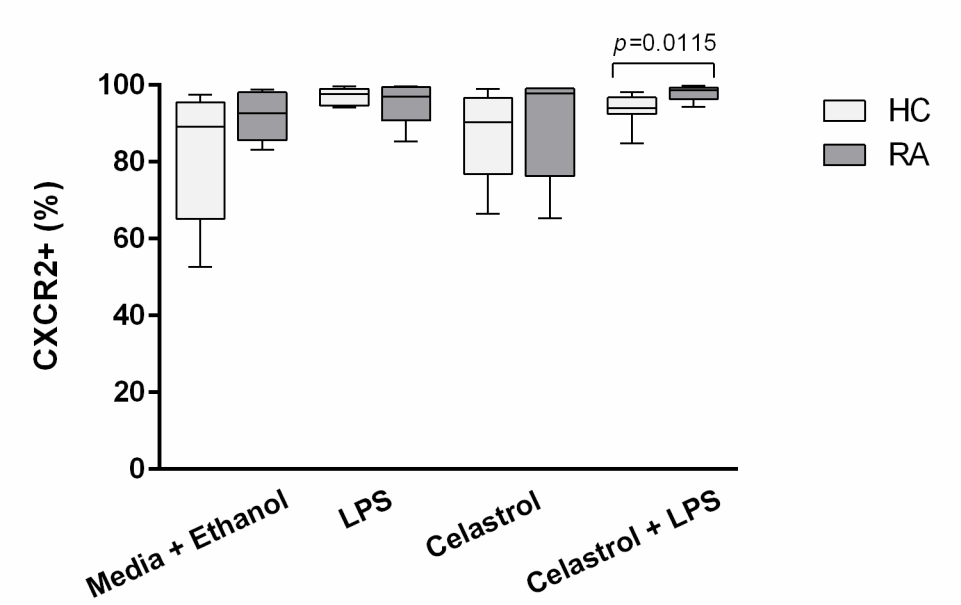

Figure 36 – Frequency of CXCR2+ cells on total CD66b+ cells in chronic RA patients tested with Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

When tested with Media + Ethanol (p=0.0193) and Celastrol (p=0.0280) conditions, had significantly increased expression of CXCR2 MFI values on total CD66b+ cells in comparison to healthy controls (**Figure 37**).

Both in the levels of CXCR2+ cells and in MFI values, a increasing linear trend is observed in all condition, except LPS. It was observed that LPS seems to decrease the CXCR2 expression in chronic RA patients.

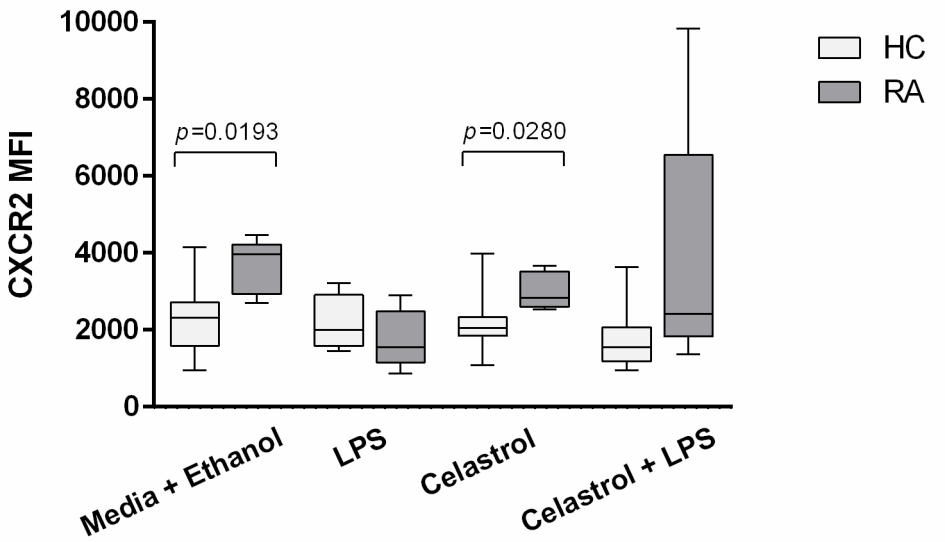

Figure 37 – Expression of CXCR2 (MFI) cell marker on CD66b+ cells on chronic RA patients tested with Media + Ethanol and Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood granulocytes cells from healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

3.3.3. T CELLS

❖ *Effect of celastrol on T cell subpopulations*

Total CD3+ T cells and the main T cell subpopulations (CD4+ and CD8+)

The analysis of the frequency of total CD3+ T cells has revealed that no statistically significant differences were found, when comparing chronic RA patients with healthy controls (data not shown). Additionally, there were no significant differences in the levels of the main T cell subpopulations (T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+)), when comparing chronic RA patients with healthy controls (data not shown).

T helper cell subpopulations (CD3+CD4+)

Regarding to the analysis of the T helper (CD3+CD4+) cell subpopulations, no significant differences were found in the frequency of the cell subpopulations analyzed based on the CD45RO and HLA-DR expression, when comparing chronic RA patients with healthy controls (data not shown), except in naïve activated (CD45RO-HLA-DR+) T cells.

Specifically, it was observed that leukocytes from chronic RA patients tested with Celastrol (p=0.0010) condition, had a significantly increased frequency of naïve activated T cells (CD45RO-HLA-DR+) in comparison to healthy controls (**Appendix III.6**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

According to the CD25 and Foxp3 expression, there were no significant differences in the levels of the regulatory T (Treg) cells (CD25+Foxp3+ subset), when comparing chronic RA patient with healthy controls (data not shown).

Additionally, no significant differences were found in the levels of CD25 subpopulations of regulatory T (Treg) cells), when comparing chronic RA patients with healthy controls (data not shown), except in $CD25^{bright}$ regulatory T (Treg) cells.

Specifically, it was observed that leukocytes from chronic RA patients tested with Celastrol (p=0.0186) condition, had a significantly increased frequency of $CD25^{bright}$

regulatory T (Treg) cells (CD4+CD25^{bright}) in comparison to healthy controls (**Appendix III.7**). An increasing linear downward is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Furthermore, there were no significant differences in the levels of the T helper cell subpopulations based on the CD45RO and CCR7 expression, when comparing chronic RA patients with healthy controls (data not shown), except in effector memory T cells (CD45RO+CCR7-).

Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0393) and Celastrol (p=0.0280) conditions, had significantly reduced frequencies of effector memory T cells in comparison to healthy controls (**Appendix III.8**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Additionaly, significant differences were found in the levels of central memory and effector memory T cell subpopulations, when comparing chronic RA patients with healthy controls, except in central memory Th17-like cells (CD45RO+CCR7+CXCR3-CCR6+) (data not shown).

Specifically, regarding to the central memory (CD45RO+CCR7+) T cell subpopulations, it was observed that leukocytes from chronic RA patients tested with Celastrol (p=0.0260) and Celastrol + LPS (p=0.0080) conditions, had significantly increased frequencies of Th1-like cells (CXCR3+CCR6-) in comparison to healthy controls (**Appendix III.9**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. When tested with Media + Ethanol (p=0.0020), LPS (p=0.0043), Celastrol (p=0.0005) and Celastrol + LPS (p=0.0007) conditions, it was observed significantly reduced frequencies of Th2-like cells (CXCR3-CCR6-) in comparison to healthy controls (**Appendix III.10**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Regarding to the effector memory (CD45RO+CCR7-) T cell subpopulations, it was observed that leukocytes from chronic RA patients tested with LPS (p=0.0303), Celastrol (p=0.0047) and Celastrol + LPS (p=0.0023) conditions, had significantly increased frequencies of Th1-like cells (CXCR3+CCR6-) in comparison to healthy controls

(**Appendix III.11**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. When tested with Celastrol + LPS (p=0.0047) condition, it was observed a significantly reduced frequency of Th2-like cells (CXCR3-CCR6-) in comparison to healthy controls (**Appendix III.12**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. When tested with Media + Ethanol (p=0.0206), LPS (p=0.0087), Celastrol (p=0.0047) and Celastrol + LPS (p=0.0080) conditions, it was observed significantly reduced frequencies of Th17-like cells (CXCR3-CCR6+) in comparison to healthy controls (**Appendix III.13**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

T cytotoxic cell subpopulations (CD3+CD8+)

Regarding to the analysis of the T cytotoxic (CD3+CD8+) cell subpopulations, no significant differences were found in the frequency of the cell subpopulations analyzed (naïve (CD45RO-HLA-DR-), naïve activated (CD45RO-HLA-DR+), memory (CD45RO+HLA-DR-) and memory activated (CD45RO+HLA-DR+) T cytotoxic cells), when comparing chronic RA patients with healthy controls (data not shown).

- *Effect of celastrol on T cell markers*

HLA-DR

Regarding to the analysis of HLA-DR, significant differences were found in HLA-DR MFI values on total CD3+ T cells and the main T cell subpopulations (T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+)) analyzed, when comparing chronic RA patients with healthy controls.

Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0043), LPS (p=0.0173), Celastrol (p=0.0030) and Celastrol + LPS (p=0.0047) conditions, had significantly increased expression of HLA-DR MFI cell marker on total CD3+ T cells in comparison to healthy controls (**Figure 38**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

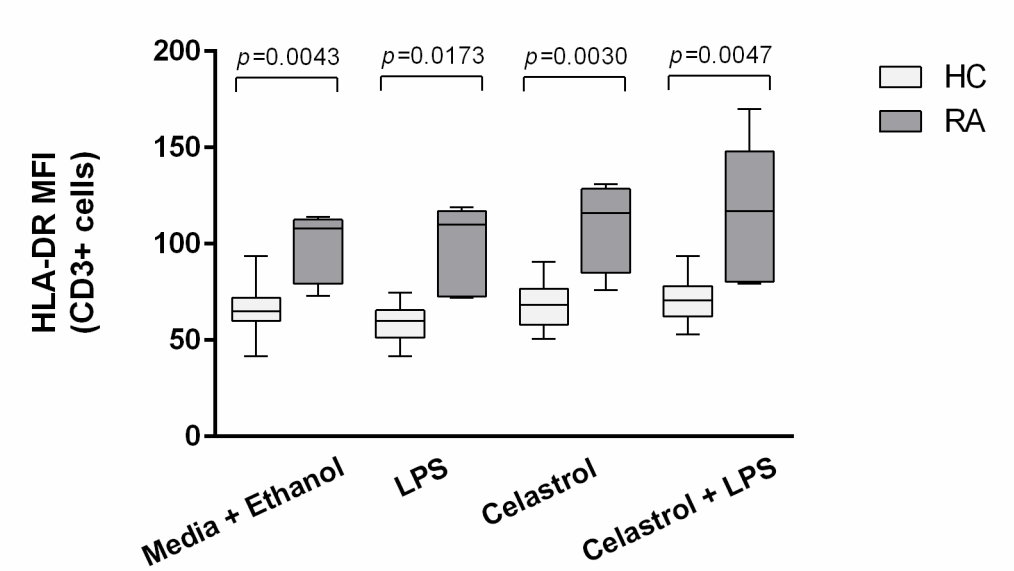

Figure 38 – Expression of HLA-DR (MFI) cell marker on total CD3+ T cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Also, when tested with Media + Ethanol (p=0.0013), LPS (p=0.0087), Celastrol (p=0.0013) and Celastrol + LPS (p=0.0027) conditions, had significantly increased expression of HLA-DR MFI cell marker on T helper cells (CD3+CD4+) in comparison to healthy controls (**Appendix III.14**). Once again, an increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. With Media + Ethanol (p=0.0120), Celastrol (p=0.0040) and Celastrol + LPS (p=0.0275) conditions, it was observed a significantly increased expression of HLA-DR MFI cell marker on T cytotoxic cells (CD3+CD8+) in comparison to healthy controls (**Appendix III.15**). Once again, an increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

CD40L

The analysis of the expression of CD40L has revelead that no significant differences were observed in CD40L MFI values on total CD3+ T cells and the main T cell subpopulations (T helper cells and T cytotoxic cells) analyzed, when comparing chronic RA patients with healthy controls (data not shown).

RORgT

Respectively to the RORgT expression, it was observed that leukocytes from chronic RA patients tested with Celastrol (p=0.0062) and Celastrol + LPS (p=0.0124) conditions, had significantly increased expression of RORgT MFI cell marker on T helper cells (CD3+CD4+) in comparison to healthy controls (**Appendix III.16**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Foxp3

Concerning to the expression of Foxp3, there were significant differences in Foxp3 MFI values on regulatory T (Treg) cells (CD25+Foxp3+ subset) and on CD25 subpopulations of regulatory T (Treg) cells (except in $CD25^{dim}$ Treg cells (data not shown)), when comparing chronic RA patients with healthy controls.

Particularly, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0295), Celastrol (p=0.0186) and Celastrol + LPS (p=0.0451) conditions, had significantly reduced expression of Foxp3 MFI cell marker on regulatory T (Treg) cells (CD25+Foxp3+ subset) in comparison to healthy controls (**Appendix III.17**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

In addition, when tested with Media + Ethanol (p=0.0451), Celastrol (p=0.0451) and Celastrol + LPS (p=0.0295) conditions, it was observed a significantly reduced expression of Foxp3 MFI cell marker on $CD25^{int}$ Treg cells (CD4+$CD25^{int}$) in comparison to healthy

controls (**Appendix III.18**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

With Media + Ethanol (p=0.0109), LPS (p=0.0455), Celastrol (p=0.0272) and Celastrol + LPS (p=0.0295) conditions, it was observed a significantly reduced expression of Foxp3 MFI cell marker on $CD25^{bright}$ Treg cells ($CD4+CD25^{bright}$) in comparison to healthy controls (**Appendix III.19**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

CCR7

The analysis of CCR7 has revelead that no significant differences were observed in CCR7 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+), when comparing chronic RA patients with healthy controls (data not shown).

CCR6

Regarding to the analysis of CCR6, there were no significant differences in CCR6 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+), when comparing chronic RA patients with healthy controls (data not shown).

CXCR3

Respectively to the CXCR3 expression, there were significant differences in CXCR3 MFI values on total CD3+ T cells and on T helper cells (CD3+CD4+), when comparing chronic RA patients with healthy controls.

Specifically, it was observed that leukocytes from chronic RA patients tested with Celastrol + LPS (p=0.0077) condition, had a significantly increased expression of CXCR3 MFI cell marker on total CD3+ T cells in comparison to healthy controls (**Figure 39**).

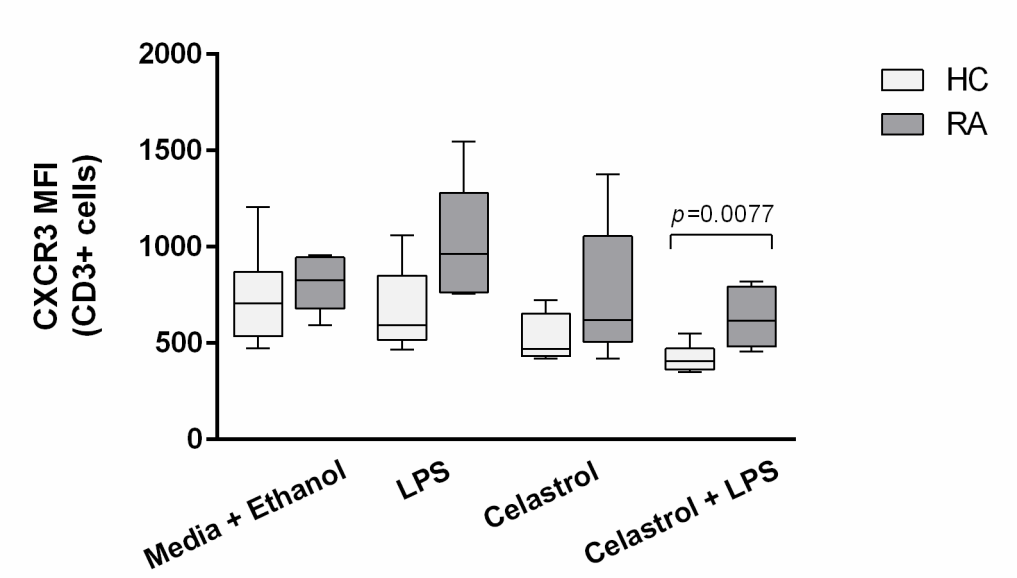

Figure 39 – Expression of CXCR3 (MFI) cell marker on total CD3+ T cells in chronic RA patients tested with Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

In addition, when tested with Media + Ethanol (p=0.0260), LPS (p=0.0303), Celastrol (p=0.0080) and Celastrol + LPS (p=0.0030) conditions, it was observed a significantly increased expression of CXCR3 MFI cell marker on T helper cells (CD3+CD4+) in comparison to healthy controls (**Appendix III.20**).

An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

3.3.4. B CELLS

❖ *Effect of celastrol on B cell subpopulations*

Total CD19+ B cells and B cell subpopulations

The analysis of the frequency of total CD19+ B cells has revealed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0027), Celastrol (p=0.0127) and Celastrol + LPS (p=0.0080) conditions, had significantly reduced frequencies of total CD19+ B cells in comparison to healthy controls (**Figure 40**). However, since a downward trend seems to be linear in all tested conditions in chronic RA patients, there being a decrease of CD19+ B cell levels in all conditions, we assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

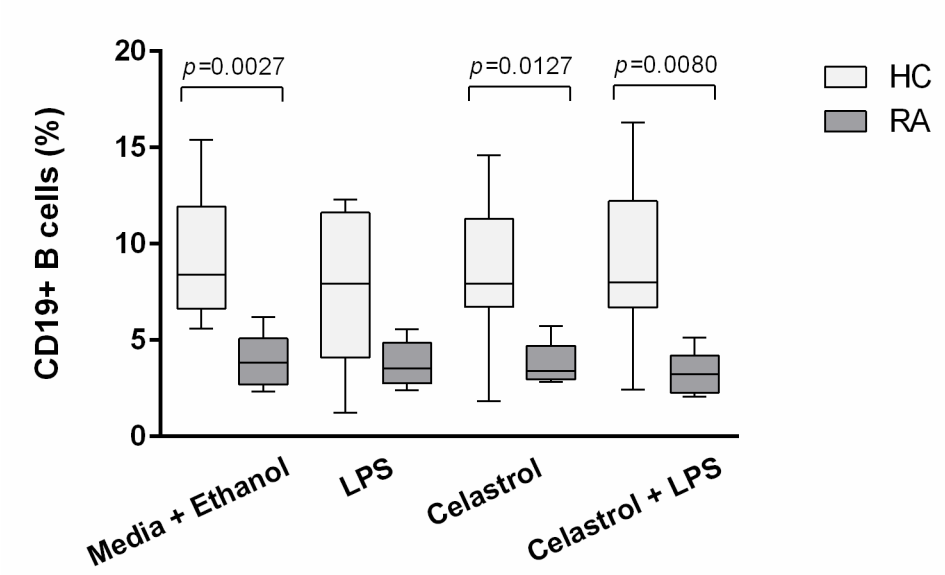

Figure 40 – Frequency of total CD19+ B cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Additionally, there were no significant differences in the levels of the B cell subpopulations (naïve, pre-switch memory, post-switch memory, double-negative B cells and plasmablasts), when comparing chronic RA patients with healthy controls (data not shown), except in transitional B cells (IgD+CD38++). Herein, it was observed that leukocytes from chronic RA patients tested with LPS (p=0.0173), Celastrol (p=0.0193) and

Celastrol + LPS (p=0.0280) conditions, had significantly reduced frequencies of transitional B cells in comparison to healthy controls (**Appendix IV.14**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

- *Effect of celastrol on B cell markers*

BAFF-R

The analysis of the frequency of BAFF-R+ cells on total CD19+ B cells has revealed that statistically significant differences were found, when comparing chronic RA patients with healthy controls. However, there were no significant differences in the levels of BAFF-R+ cells in all B cell subpopulations (except in pre-switch memory B cells) analyzed, when comparing chronic RA patients with healthy controls (data not shown).

Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0007) and Celastrol + LPS (p=0.0080) conditions, had significantly reduced frequencies of BAFF-R+ cells on total CD19+ B cells in comparison to healthy controls (**Figure 41**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

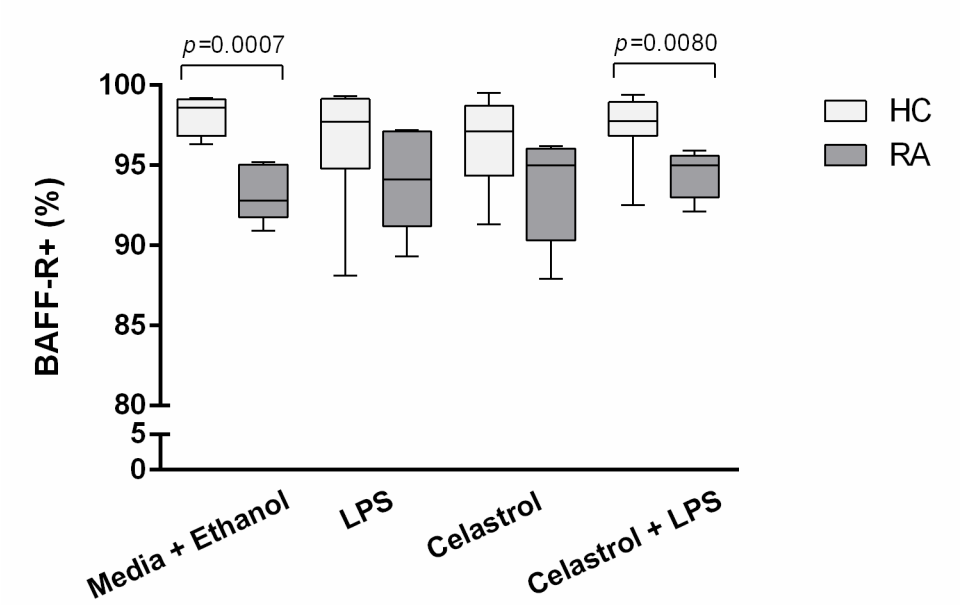

Figure 41 – Frequency of BAFF-R+ cells on total CD19+ B cells in chronic RA patients tested with Media + Ethanol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Similarly, when tested with Media + Ethanol (p=0.0020), LPS (p=0.0317), Celastrol (p=0.0113) and Celastrol + LPS (p=0.0210) conditions, had significantly reduced frequencies of BAFF-R+ cells on pre-switch memory (IgD+CD27+) B cells in comparison to healthy controls (**Appendix IV.15**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Furthermore, significant differences were found in BAFF-R MFI values on total CD19+ B cells and all B cell subpopulations analyzed, when comparing chronic RA patients with healthy controls.

Particularly, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0027), LPS (p=0.0173), Celastrol (p=0.0027) and Celastrol + LPS (p=0.0027) conditions, had significantly reduced expression of BAFF-R MFI cell marker on total CD19+ B cells in comparison to healthy controls (**Figure 42**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

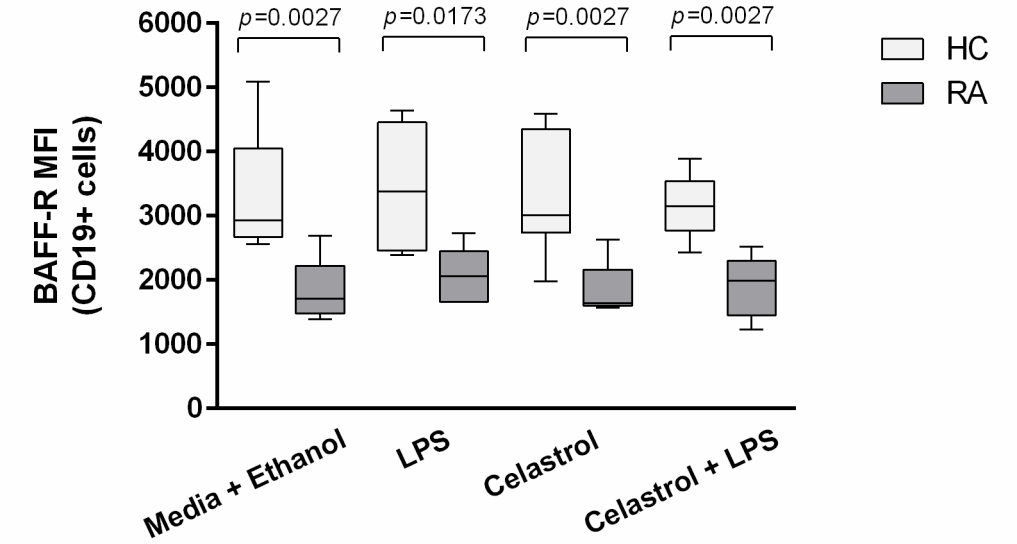

Figure 42 – Expression of BAFF-R (MFI) cell marker on total CD19+ B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Similarly, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0013), LPS (p=0.0173), Celastrol (p=0.0027) and Celastrol + LPS (p=0.0013) conditions, had significantly reduced expression of BAFF-R MFI cell marker on naïve B cells (IgD+CD27-) in comparison to healthy controls (**Appendix IV.16**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. Also, when tested with Media + Ethanol (p=0.0047), LPS (p=0.0159), Celastrol (p=0.0070) and Celastrol + LPS (p=0.0020) conditions, had significantly reduced expression of BAFF-R MFI cell marker on pre-switch memory (IgD+CD27+) B cells in comparison to healthy controls (**Appendix IV.17**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. With Media + Ethanol (p=0.0127), LPS (p=0.0317), Celastrol (p=0.0070) and Celastrol + LPS (p=0.0120) conditions, it was observed a significantly reduced expression of BAFF-R MFI cell marker on post-switch memory (IgD-CD27+) B cells in comparison to healthy controls (**Appendix IV.18**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. With Media + Ethanol

(p=0.0047), LPS (p=0.0079) and Celastrol + LPS (p=0.0120) conditions, it was observed a significantly reduced expression of BAFF-R MFI cell marker on double-negative (IgD-CD27-) B cells in comparison to healthy controls (**Appendix IV.19**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

FcgRIIB

Regarding to the analysis of FcgRIIB, no significant differences were found in FcgRIIB MFI values on total CD19+ B cells and all B cell subpopulations analyzed, when comparing chronic RA patients with healthy controls (data not shown).

CD38

The analysis of the expression of CD38 has revelead that no significant differences were observed in CD38 MFI values on total CD19+ B cells and all B cell subpopulations analyzed (except in post-switch memory and double-negative B cells), in all tested conditions, when comparing chronic RA patients with healthy controls (data not shown). However, it was observed that leukocytes from chronic RA patients tested with LPS (p=0.0303) and Celastrol (p=0.0183) conditions, had significantly reduced expression of CD38 MFI cell marker on post-switch memory (IgD-CD27+) B cells, in comparison to healthy controls (**Appendix IV.20**); and when tested with Media + Ethanol (p=0.0047), LPS (p=0.0173), Celastrol (p=0.0027) and Celastrol + LPS (p=0.0040) conditions, had significantly reduced expression of CD38 MFI cell marker on double-negative (IgD-CD27-) B cells, in comparison to healthy controls (**Appendix IV.21**). A linear downward trend is observed in all tested conditions of post-switch memory and double-negative B cells, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

CD21

Respectively to the CD21 expression, it was revealed that statistically significant differences were found in the levels of CD21+ cells on total CD19+ B cells and all B cell subpopulations (except in post-switch memory B cells), when comparing chronic RA patients with healthy controls. Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0010), LPS (p=0.0303), Celastrol (p=0.0053) and Celastrol + LPS (p=0.0127) conditions, had significantly reduced frequencies of CD21+ cells on total CD19+ B cells in comparison to healthy controls (**Figure 43**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

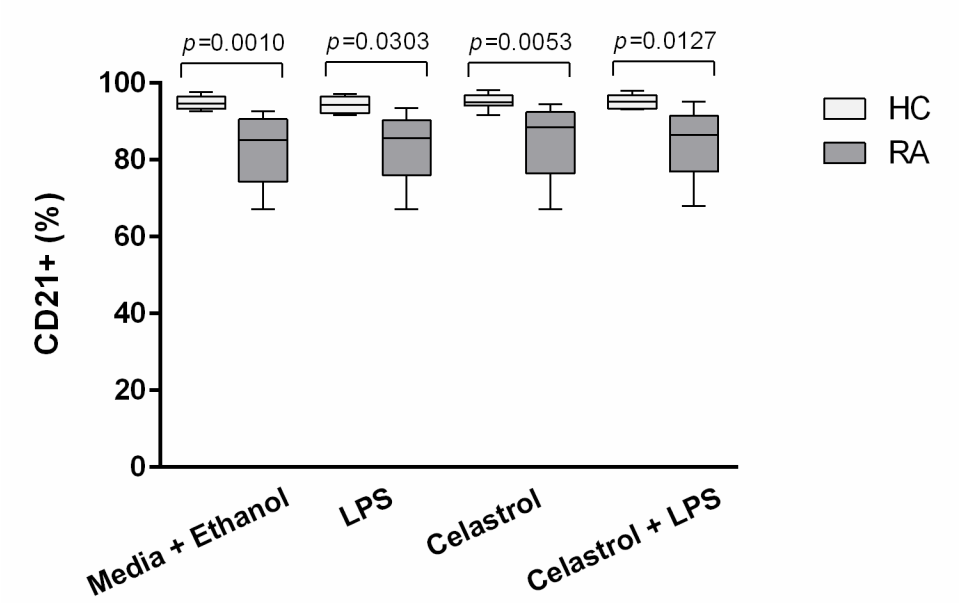

Figure 43 – Frequency of CD21+ cells on total CD19+ B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for p-values<0.05 according to the Mann-Whithey tests.

Similarly, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0097), Celastrol (p=0.0436) and Celastrol + LPS (p=0.0117) conditions, had significantly reduced frequencies of CD21+ cells on naïve B cells (IgD+CD27-) in comparison to healthy controls (**Appendix IV.22**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences

observed are due to the disease phenotype and not to the effect of the added stimuli. Also, when tested with Media + Ethanol (p=0.0023), LPS (p=0.0159), Celastrol (p=0.0013) and Celastrol + LPS (p=0.0010) conditions, had significantly reduced frequencies of CD21+ cells on pre-switch memory (IgD+CD27+) B cells in comparison to healthy controls (**Appendix IV.23**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Furthermore, no significant differences were found in CD21 MFI values on total CD19+ B cells and all B cell subpopulations (except in pre-switch memory B cells) analyzed, when comparing chronic RA patients with healthy controls.

Particularly, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0047), LPS (p=0.0079), Celastrol (p=0.0007) and Celastrol + LPS (p=0.0007) conditions, had significantly reduced expression of CD21 MFI cell marker on pre-switch memory B cells (IgD+CD27+) in comparison to healthy controls (**Appendix IV.24**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

$CD21^{low}CD38^{low}$

Concerning to the expression of $CD21^{low}CD38^{low}$, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0007), LPS (p=0.0317), Celastrol (p=0.0013) and Celastrol + LPS (p=0.0047) conditions, had significantly increased frequencies of $CD21^{low}CD38^{low}$+ cells on total CD19+ B cells in comparison to healthy controls (**Figure 44**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

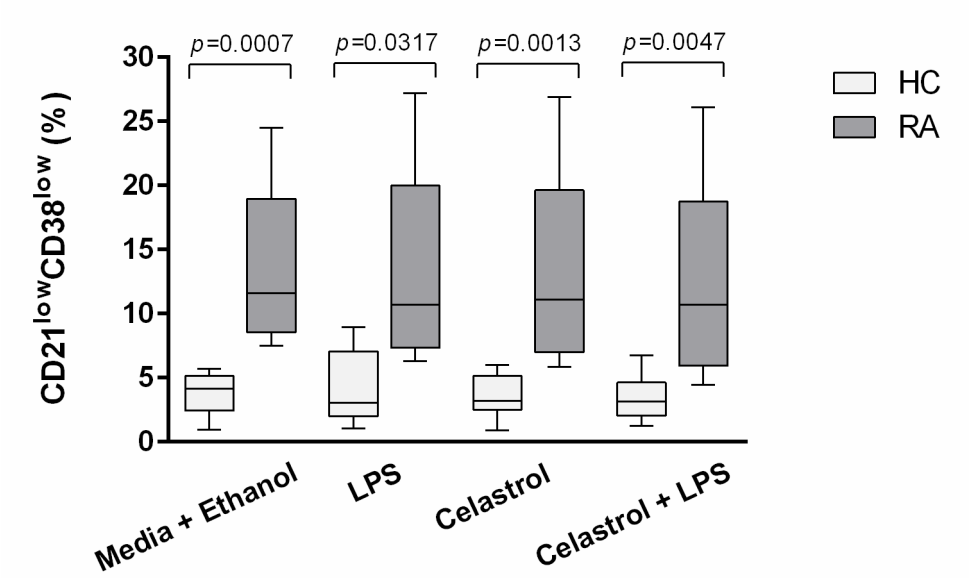

Figure 44 – Frequency of CD21lowCD38low+ cells on total CD19+ B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

HLA-DR

The analysis of the expression of HLA-DR has revelead that statistically significant differences were found in the levels of HLA-DR+ cells on total CD19+ B cells and all B cell subpopulations (except in naïve B), when comparing chronic RA patients with healthy controls.

Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0137), Celastrol (p=0.0007) and Celastrol + LPS (p=0.0013) conditions, had significantly reduced frequencies of HLA-DR+ cells on total CD19+ B cells in comparison to healthy controls (**Figure 45**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

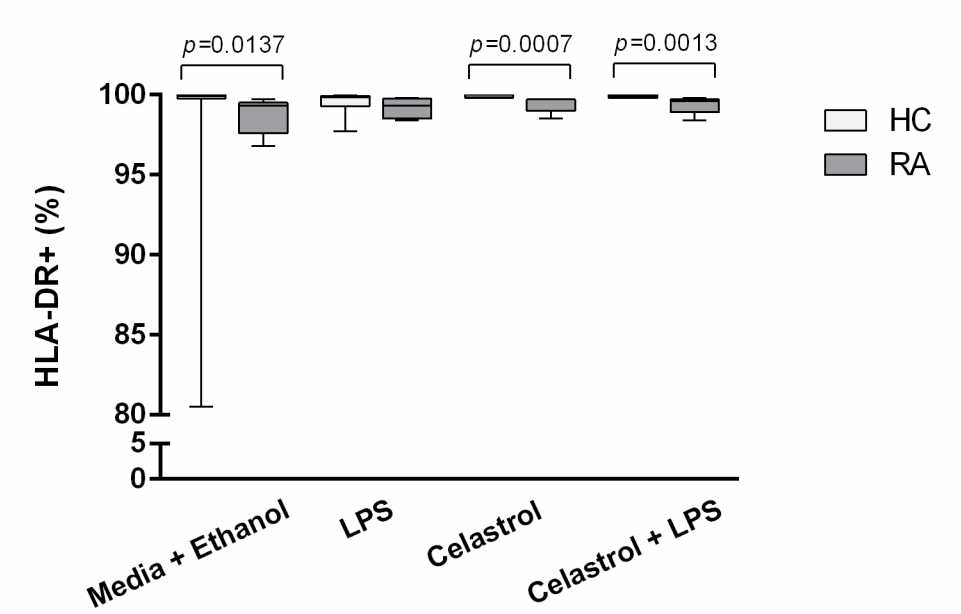

Figure 45 – Frequency of HLA-DR+ cells on total CD19+ B cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

Similarly, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0050), LPS (p=0.0238) and Celastrol + LPS (p=0.0007) conditions, had significantly reduced frequencies of HLA-DR+ cells on pre-switch memory B cells (IgD+CD27+) in comparison to healthy controls (**Appendix IV.25**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli. Also, when tested with Celastrol (p=0.0483) condition, had significantly reduced frequency of HLA-DR+ cells on post-switch memory B cells (IgD-CD27+) in comparison to healthy controls (**Appendix IV.26**). Once again, a linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

Furthermore, no significant differences were found in HLA-DR MFI values on total CD19+ B cells and all B cell subpopulations analyzed, when comparing chronic RA patients with healthy controls.

RANKL

Regarding to the analysis of RANKL, there were no significant differences in the levels of RANKL+ cells on total CD19+ B cells, when comparing chronic RA patients with healthy controls (data not shown). However, it was revealed that statistically significant differences were found in the levels of RANKL+ cells on all B cell subpopulations (except in naïve B cells and pre-switch memory B) analyzed, when comparing chronic RA patients with healthy controls.

Specifically, it was observed that leukocytes from chronic RA patients tested with Celastrol (p=0.0025) condition, had significantly increased frequency of RANKL+ cells on post-switch memory B cells (IgD-CD27+) in comparison to healthy controls (**Appendix IV.27**). Also, with Celastrol (p=0.0485) condition, it was observed a significantly increased frequency of RANKL+ cells on double-negative B cells (IgD-CD27-) in comparison to healthy controls (**Appendix IV.28**).

Both in the frequencies of RANKL+ on post-switch memory B cells (IgD-CD27+) and on double-negative B cells (IgD-CD27-), an increasing linear trend is observed, but celastrol seems indicate that reduces the frequencies of these subpopulations in healthy controls.

Furthermore, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0109) and Celastrol + LPS (p=0.0420) conditions, had significantly reduced expression of RANKL MFI values on total CD19+ B cells in comparison to healthy controls (**Figure 46**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

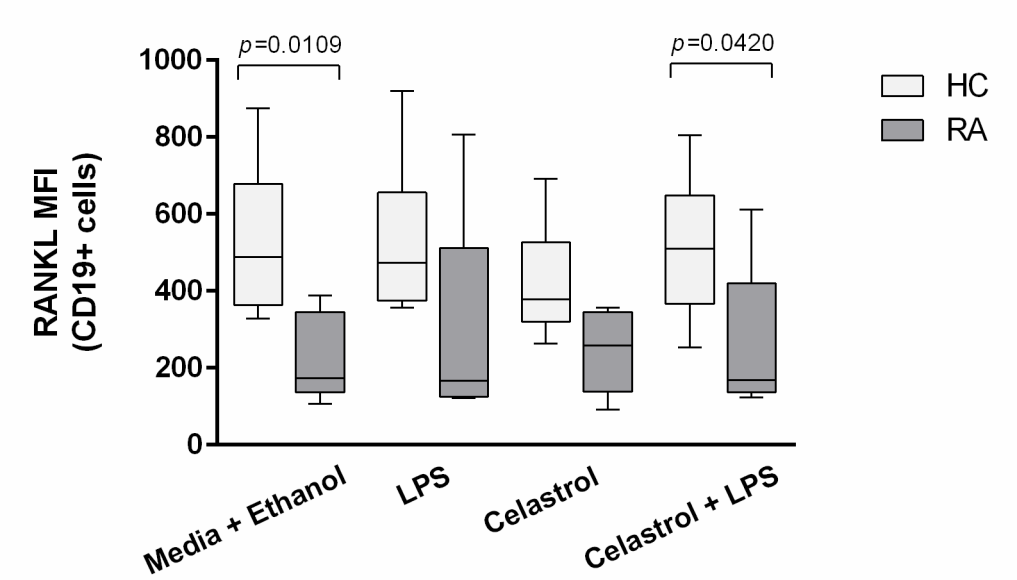

Figure 46 – Expression of RANKL (MFI) cell marker on total CD19+ B cells in chronic RA patients tested with Media + Ethanol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

However, no significant differences were found in RANKL MFI values on all B cell subpopulations (except in naïve B cells) analyzed, when comparing chronic RA patients with healthy controls (data not shown).

Specifically, it was observed that leukocytes from chronic RA patients tested with Media + Ethanol (p=0.0031), Celastrol (p=0.0186), Celastrol + LPS (p=0.0120) conditions, had significantly reduced expression of RANKL MFI values on naïve B cells (IgD+CD27-) in comparison to healthy controls (**Appendix IV.29**). A linear downward trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

CD95

Respectively to the CD95 expression, there were no significant differences in the levels of CD95+ cells on total CD19+ B cells and all B cell subpopulations analyzed, when comparing chronic RA patients with healthy controls (data not shown).

Furthermore, no significant differences were found in CD95 MFI values on total CD19+ B cells and all B cell subpopulations (except in double-negative B cells) analyzed, when comparing chronic RA patients with healthy controls.

Particularly, it was observed that leukocytes from chronic RA patients tested with Celastrol (p=0.0240) condition, had significantly increased expression of CD95 MFI cell marker on double-negative B cells (IgD-CD27-) in comparison to healthy controls (**Appendix IV.30**). An increasing linear trend is observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype and not to the effect of the added stimuli.

CHAPTER V – DISCUSSION AND CONCLUSIONS

In the present study the effect of celastrol, a bioactive component of the chinese plant *Tripterygium wilfordii*, on primary human leukocytes (monocytes, granulocytes, B and T lymphocytes and their subsets) activation, maturation, survival and apoptosis was studied using peripheral blood samples collected from chronic RA patients and healthy controls.

For this purpose, we first proceeded to the optimization of experimental conditions. Leukocytes from healthy controls (*n*=4) were incubated for different incubation times with celastrol and stimulated with LPS, at a range of concentrations, to assess the optimal compounds concentrations and duration of the incubation. After incubation, cell viability was assessed using alarmarBlue® by spectrophotometry assay, and an immunophenotyping characterization of monocytes, granulocytes, B and T cells was performed by Flow Cytometry. Among the tested conditions, we found that incubation for 4h with celastrol 0,3 μM and LPS 10 μg/mL was the condition that showed less lymphocyte's, monocyte's and granulocyte's cell death, appearing to be the best condition to be tested in the following experiments. Next, we studied the effect of celastrol in WBCs of the recruited chronic RA patients (*n*=5) and healthy controls (*n*=10), using the pre-optimized experimental conditions. Of note, LPS was used as a non-specific stimulant to activate the peripheral WBCs.

Macrophages are a hallmark of inflammation and, the abundance of these cells in RA joints mirrors disease activity (Haringman JJ et al., 2005). After its activation are thought to be major contributors to pro-inflammatory cytokine production (Jovanovic DV et al., 1998; Clavel C et al., 2008) and joint destruction through osteoclastogenesis (Ma Y et al., 2005). These cells migrate as monocytes from peripheral blood, infiltrate and accumulate in the synovium (Ma Y et al., 2005), where they can be differentiated into macrophages and in osteoclasts (Auffray C et al., 2009; Komano Y et al., 2006; Gordon S et al., 2005).

In the present study, we revealed that celastrol was able to restore the frequencies of total CD14+ cells to basal levels in monocytes from healthy controls, but no significant differences were observed in chronic RA patients, maybe due to the limiting effect of LPS. We assumed as an incongruent data that occurred perhaps due to the occurrence of monocyte adhesion on cell culture plates aggravated with the LPS addition, leading a significant cell loss and consequently, an inconsistent analysis.

Shinohara S et al., showed that the expression of CD14 was upregulated on peripheral blood monocytes from patients with active RA (Shinohara S et al., 1992).

In fact, in our study, when comparing healthy controls with chronic RA patients, the frequencies of total CD14+ cells were similar at basal levels, but increased in chronic RA patients when leukocytes were stimulated with LPS. However, celastrol seems to reduce the frequencies of total CD14+ cells in chronic RA patients.

It should not be overlooked the limiting effect of LPS and the reduced cohort of patients (n=5).

Three monocytes subpopulations, have been described in humans (Wong KL et al., 2012). However, LPS addition led to an increased adhesion of monocytes on plate wells, and consequently a significant loss of cells was observed, leading to a number of inconsistent results in comparison to the literature. Thus, the analysis of all monocyte subpopulations was excluded in order not to lead to wrong interpretations.

Additionaly, in this study we have shown a decrease in the expression of surface markers on monocytes, both in leukocytes from healthy controls and chronic RA patients tested with celastrol compound.

Previous studies have shown that monocytes have an activated phenotype in RA with increased expression of several surface markers, such as CD16 (monocyte activation marker), HLA-DR (involved in antigen presentation to CD4+ T cells), CD86 (costimulatory molecule of T cells) and CD115 (involved in monocyte differentiation) (Shinohara S et al., 1992; Kawanaka N et al., 2002; Thomas R et al., 1996; Iguchi T et al., 1986).

Specifically, in our study we have reported that celastrol (after LPS stimulation) was able to diminish both the frequencies and expression MFI levels of CD16 on total CD14+ cells in monocytes from healthy controls, but no significant differences were observed in chronic RA patients and when comparing healthy controls with chronic RA patients.

We have shown that celastrol was able to restore the expression MFI levels of HLA-DR on total CD14+ cells to basal levels in monocytes from healthy controls. However, no significant differences were observed in chronic RA patients. When comparing healthy controls with chronic RA patients, it was observed that the expression MFI levels of HLA-DR on total CD14+ cells were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

In addition, the frequencies and expression MFI levels of CD86 on total CD14+ cells in healthy controls appeared to be restored to basal levels with celastrol. However, in chronic

RA patients, celastrol (after LPS stimulation) was able to decrease the frequencies of CD86 on total CD14+ cells. When comparing healthy controls with chronic RA patients, it was observed that the frequencies and expression MFI levels of CD86 on total CD14+ cells were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

Regarding to CD115, we have reported that celastrol (after LPS stimulation) was able to diminish both the frequencies and expression MFI levels of CD115 on total CD14+ cells in healthy controls. Moreover, in chronic RA patients, celastrol was able to decrease the frequencies of CD115 on total CD14+ cells. However, no significant differences were observed when comparing healthy controls with chronic RA patients.

Granulocytes are the most abundant leukocytes and include neutrophils, which are the first cell type to infiltrate the synovium and arrive at sites of inflammation (Nathan C et al., 2006). In RA, the mechanisms of neutrophil activation, recruitment and apoptosis are altered (Weinmann P et al., 2007) and it has been described that these phagocytic cells when activated play a crucial role since the early phase of the disease (Cascão R et al., 2010), as they are able to modulate the function of other immune cells and contribute to the perpetuation of an initial inflammatory response.

Granulocytes when activated express CD66b (population marker), CD11b (granulocyte activation marker), CD16 (involved in the immunophagocytosis; specifically, $CD16^{high}$ - nonapoptotic granulocytes), CD15 (activation and adhesion marker), CD62L (granulocyte adhesion marker) and CXCR2 (major granulocyte chemoattractant receptor) surface markers.

Given the proposed role of granulocytes in the pathogenesis of RA, we found that the celastrol had no significant effect on total CD66b+ cells in granulocytes, both in healthy controls and chronic RA patients. When comparing healthy controls with chronic RA patients, it was observed that the frequencies of total CD66b+ cells were increased in chronic RA patients.

In our study we have reported that celastrol was able to restore the expression MFI levels of CD11b on total CD66b+ cells to basal levels in granulocytes from healthy controls and chronic RA patients, but no significant differences were observed when comparing healthy controls with chronic RA patients.

In addition, no significant differences were observed in the frequencies and expression MFI levels of $CD16^{high}$ and $CD16^{low}$ both in healthy controls and chronic RA patients,

however, when comparing healthy controls with chronic RA patients it was observed that the frequencies of $CD16^{high}$ and $CD16^{low}$ on total CD66b+ cells were increased and reduced, respectively, in chronic RA patients.

We have shown that no significant differences were observed in the frequencies and expression MFI levels of CD15 both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients it was observed that the frequencies of CD15 on total CD66b+ cells were increased, in chronic RA patients, but no effect of celastrol on these cells was observed..

Regarding CD62L, we have reported that celastrol was able to diminish the expression MFI levels of CD62L on total CD66b+ cells in healthy controls, but no significant differences were observed in chronic RA patients. Morever, when comparing healthy controls with chronic RA patients, it was observed that the expression MFI levels of CD62L on total CD66b+ cells were reduced in chronic RA patients.

Lastly, the frequencies of CXCR2 on total CD66b+ cells in healthy controls appeared to be restored to basal levels with celastrol, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients, it was observed that the frequencies and expression MFI levels of CXCR2 on total CD66b+ cells were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

For a long time, RA has been considered as a T-cell driven disease (Boissier MC et al., 2008). Reports have implicated Th17 cells as important effectors in RA pathogenesis due to the overexpression of IL-17, which is associated with an aggravation of inflammation and joint damage (Lubberts E et al., 2005; Chabaud M et al., 1999). Cytokines that support Th17 differentiation, suppress Treg cells polarization, and consequently shift T cell homeostasis towards inflammation.

In this study, we have shown that celastrol had no significant effect on total CD3+ T cells, both in healthy controls and chronic RA patients, not even when comparing healthy controls with chronic RA patients.

In this study, the main T cell subpopulations (CD4+ and CD8+ T cells - Thelper and Tcytotoxic cells, respectively) and their subsets were analyzed. Several studies have observed reduced circulating Treg cell percentages in RA compared to healthy controls, and have pointed the importance of Th17/Treg cell imbalance in the pathogenesis of RA (Niu Q

et al., 2012; Kawashiri SY et al., 2011; Sempere-Ortells JM et al., 2009).

Regarding to the frequencies of T cell subpopulations studied, we have reported that celastrol was able to restore the frequencies of naïve activated T helper cells (CD45RO-HLA-DR+) to basal levels in T cells from healthy controls, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this population were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

Additionally, it was observed that celastrol had no significant effect on memory activated T helper cells (CD45RO+HLA-DR+) in healthy controls, but it was able to increase the frequencies of this subpopulation in chronic RA patients. However, no significant differences were observed when comparing healthy controls with chronic RA patients.

We have found that celastrol had no significant effect on $CD25^{bright}$ Treg cells ($CD4+CD25^{bright}$), both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this population were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

Regarding to the central memory Th1-like cells (CXCR3+CCR6-), we have shown that celastrol had no significant effect on this subpopulation, both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this subpopulation were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

In addition, the frequencies of central memory Th2-like cells (CXCR3-CCR6-) in healthy controls appeared to be increased with celastrol, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this subpopulation were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

Also, we have found that celastrol had no significant effect on effector memory T helper cells (CD45RO+CCR7-), both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this subpopulation were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

We have reported that celastrol had no significant effect on effector memory Th1-like cells (CXCR3+CCR6-), both in healthy controls and chronic RA patients, however, when

comparing healthy controls with chronic RA patients, it was observed that the frequencies of this subpopulation were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

In addition, the frequencies of effector memory Th2-like cells (CXCR3-CCR6-) in healthy controls appeared to be increased with celastrol, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this subpopulation were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

We have found that celastrol had no significant effect on effector memory Th17-like cells (CXCR3-CCR6+), both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of this population were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

In the current study, we noticed an effect of celastrol on several cellular markers on T cells, concerning to the T cell activation (HLA-DR), Th17 cells differentiation (RORgT), suppressive activity of Treg cells (Foxp3) and chemotaxis of memory Th1 cells (CXCR3).

Particularly, we have shown that celastrol had no significant effect in the expression MFI levels of HLA-DR both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients it was observed that the expression of MFI levels of HLA-DR on total CD3+ T cells and T helper (CD3+CD4+) and T cytotoxic (CD3+CD8+) cells were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

Also, we have reported that celastrol had no significant effect in the expression MFI levels of RORgT both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients it was observed that the expression of MFI levels of RORgT on T helper cells (CD3+CD4+) were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

In addition, we have found that celastrol had no significant effect in the expression MFI levels of Foxp3 both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients it was observed that the expression of MFI levels of Foxp3 on T reg cells (CD25+Foxp3+ subset), $CD25^{int}$ ($CD4+CD25^{int}$) and $CD25^{bright}$ ($CD4+CD25^{bright}$) Treg cells were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

Among CD4+CD25+ cells, only those expressing large amounts of CD25

(CD4+CD25^{bright}) exert suppressive effects. Cells expressing intermediate amounts of CD25 (CD4+CD25^{int}), in contrast, are effector CD4 cells (Mottonen M et al., 2005). In RA, it has been demonstrated that the expression of Foxp3 by Treg cells is diminished which affects the ability of Treg cells to suppress effector T-cell proliferation and cytokine secretion (Valencia X et al., 2006).

In concordance with the literature, it was observed that Foxp3 expresssion (MFI) of Treg cells was significantly reduced in RA patients in comparison with healthy individuals, however, no effect was found when this cell population was in the presence of celastrol.

Lastly, the expression MFI levels of CXCR3 on total CD3+ T cells and T helper cells (CD3+CD4+) in healthy controls appeared to be reduced with celastrol (after LPS stimulation), but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients, it was observed that the expression MFI levels of CXCR3 on total CD3+ T cells and T helper cells (CD3+CD4+) were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

Several studies have documented the importance of B cells in RA progression through diverse mechanisms, not only by producing and releasing autoantibodies such as RF and ACPA, which activate macrophages and induce production of pro-inflammatory cytokines, but also throug their functions as APCs for T cell activation (Smolen JS et al., 2007; Kinne RW et al., 2007; Weyand CM et al., 2005; Moura RA et al., 2012).

In the present study, we found that the celastrol had no significant effect on total CD19+ B cells, both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of total CD19+ B cells were reduced in chronic RA patients, but celastrol did not seem to have significant effect on this population, since a downward linear trend was observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype/activity and not to the effect of the compound.

The main B cell subpopulations were analyzed, depending on their IgD /CD27 and IgD/CD38 expression. Abnormalities in the distribution of peripheral blood B cell subpopulations, particularly memory B cell subsets, have been reported in RA patients (Moura RA et al., 2010; Souto-Carneiro MM et al., 2009). Of note, it has been previously shown that established RA patients have significantly lower levels of peripheral blood pre-switch (IgD+CD27+) memory B cells (Souto-Carneiro MM et al., 2009), and significantly

increased levels of double-negative (IgD-CD27-) memory B cells (Moura RA et al., 2017) when compared to healthy controls.

In our study, we have shown that celastrol had no significant effect in the frequencies of all B cell subpopulations both in healthy controls and chronic RA patients. When comparing healthy controls with chronic RA patients, it was observed that the frequencies of transitional B cells (IgD+CD38++) were reduced in chronic RA patients, but celastrol did not seem to have significant effect on this population, since a downward linear trend was observed in all tested conditions, which leads us to assume that the significant differences observed are due to the disease phenotype/activity and not to the effect of the compound

In this study, several B cell markers were studied which were directly related with B cell activation (HLA-DR) and survival (BAFF-R), B cell adhesion, activation and calcium signaling (CD38), cell inhibition (FcgRIIB), complement system activation: autoimmunity-associated marker ($CD21^{low}CD38^{low}$) / immature B cells (CD21), osteoclast activation and differentiation (RANKL) and Fas-mediated apoptosis (CD95).

Particularly, we have shown that celastrol (after LPS stimulation) was able to restore the frequencies of HLA-DR on transitional B cells (IgD+CD38++) to basal levels in B cells from healthy controls, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients, it was observed that the frequencies of HLA-DR on total CD19+ B cells, pre-switch memory (IgD+CD27+) and post-switch memory (IgD-CD27+) B cells (IgD+CD38++) were reduced in chronic RA patients, but no effect of celastrol on these cells was observed

Previous studies have reported that B cells once activated increase the expression of activation markers such as HLA-DR (Feldmann M et al., 1996), in accordance with their function as APCs locally in the joints (Takemura S et al., 2001; Heldt C et al., 2003; Bowes J et al., 2008). Contrarily, in our study, it was observed a trend to reduce the HLA-DR frequency on transitional B cells (IgD+CD38++) when leukocytes are stimulated with LPS, and a restore to basal levels upon celastrol (after LPS stimulation) incubation.

Regarding BAFF-R, celastrol had no significant effect in the frequencies and expression MFI levels of BAFF-R both in healthy controls and chronic RA patients. When comparing healthy controls with chronic RA patients it was observed that the frequencies of BAFF-R on total CD19+ B cells and pre-switch memory (IgD+CD27+) B cells and the expression MFI levels on total CD19+ B cells, naïve B cells (IgD+CD27-), pre-switch memory (IgD+CD27+), post-switch memory (IgD-CD27+) and double-negative (IgD-CD27-) B cells were reduced in chronic RA patients, but no effect of celastrol on these cells

was observed.

In addition, no significant differences were observed in the expression MFI levels of CD38 both in healthy controls and chronic RA patients, however, when comparing healthy controls with chronic RA patients it was observed that the expression MFI levels of CD38 on post-switch memory (IgD-CD27+) and double-negative (IgD-CD27-) B cells were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

Regarding FcgRIIB, we have observed that celastrol was able to restore the expression MFI levels of FcgRIIB on plasmablasts (IgD-CD38++) to basal levels in healthy controls, but no significant differences were observed in chronic RA patients and when comparing healthy controls with chronic RA patients.

However, data have shown that peripheral B cells from RA patients have altered expression of FcgRIIB, potentially contributing to tolerance breakdown and development of humoral autoimmunity (Catalan D et al., 2010).

Importantly, our study has not shown the same effect of celastrol on chronic RA patients as shown in healthy controls, possibly due to reduced cohort of patiens (*n*=5) which is not sufficient to have robust results and consistent with the literature.

We have also reported that celastrol was able to restore the expression MFI levels of CD21 on total CD19+ B cells and naïve B cells (IgD+CD27-) to basal levels in healthy controls, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients it was observed that the frequencies of CD21 on total CD19+ B cells, naïve (IgD+CD27-) and pre-switch memory (IgD+CD27+) B cells were reduced in chronic RA patients, and the expression levels of CD21 on pre-switch memory (IgD+CD27+) B cells were also reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

The activation of B cells leads to reduced surface levels of CD21, as it has been shown in peripheral blood of RA patients (Masilamani M et al., 2003).

Importantly, we have shown that no significant differences were observed in the frequencies of $CD21^{low}CD38^{low}$ both in healthy controls and chronic RA patients. When comparing healthy controls with chronic RA patients, it was observed that the frequencies $CD21^{low}CD38^{low}$ on total CD19+ B cells were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

It has been published that B cells with low expression of CD21 ($CD21^{low}$ B cells) are increased in the peripheral blood of patients with RA (Isnardi I et al., 2010) and others autoimmune diseases (Wehr C et al., 2004; Nicholas MW et al., 2008), as shown in our

study. CD21^{low} B cells express elevated levels of CD19 (Erickson LD et al., 2001) and low levels of CD38 (Danzer CP et al., 2003). Isnardi et al., found that CD21^{low} B cells in were unable to induce calcium influx, become activated, or proliferate in response to B-cell receptor (Isnardi I et al., 2010).

Moreover, we have shown that celastrol was able to restore the frequencies of RANKL on total CD19+ B cells, naïve (IgD+CD27-), pre-switch memory (IgD+CD27+), post-switch memory (IgD-CD27+) B cells to basal levels and diminish the frequencies of RANKL on transitional B cells (IgD+CD38++) in healthy controls, but no significant differences were observed in chronic RA patients. However, when comparing healthy controls with chronic RA patients it was observed that the frequencies of RANKL on post-switch memory (IgD-CD27+) and double-negative (IgD-CD27-) B cells were increased in chronic RA patients, and in contrast, the expression MFI levels of RANKL on total CD19+ B cells and naïve B cells (IgD+CD27-) were reduced in chronic RA patients, but no effect of celastrol on these cells was observed.

Regarding to CD95, we have reported that celastrol was able to restore the frequencies of CD95 on naïve B cells (IgD+CD27-) and plasmablasts (IgD-CD38++) and the expression MFI levels on plasmablasts (IgD-CD38++) to basal levels, and was also able to diminish the frequencies of CD95 on transitional B cells (IgD+CD38++) and the expression MFI levels on naïve B cells (IgD+CD27-) and transitional B cells (IgD+CD38++) in healthy controls. However, no significant differences were observed in chronic RA patients, but when comparing healthy controls with chronic RA patients it was observed that the expression MFI levels of CD95 on double-negative (IgD-CD27-) B cells were increased in chronic RA patients, but no effect of celastrol on these cells was observed.

Importantly, defects in apoptosis, namely in the CD95-dependent death receptor pathway, have been demonstrated to occur in RA pathogenesis and a significant expansion of activated memory B cell populations expressing high levels of CD95 in the peripheral blood has been documented (Cantwell MJ et al., 1997; Rapetti et al., 2013).

Importantly, our study has not shown the same effect of celastrol in some cases of cell populations on chronic RA patients as shown in healthy controls, possibly due to the reduced cohort of patiens (n=5) which is not sufficient to have robust and significant results and consistent with the literature. In addition, about monocytes, it should not be overlooked the limiting effect of LPS, which produced unreliable results and incongruent data that

occurred perhaps due to the occurrence of monocyte adhesion on cell culture plates aggravated with the LPS addition, leading a significant cell loss and consequently, an inconsistent analysis.

Although the mechanisms of action of celastrol are not yet fully understood, this work brings new insights regarding to the effect of this compound on human peripheral blood leukocytes activation, maturation, survival and apoptosis both in healthy control and chronic RA patients.

Altogether, our data revealed that celastrol seems to have a stronger effect on innate immune system cells, reducing their overall activation, differentiation and migration potential. Importantly, this finding has implications for the selection of potential diseases and disease stages where this compound might be particularly effective.

To sum up, we suggest that celastrol is a promising candidate for future clinical trials in RA patients and if validated, might serve as useful adjunct/alternative to conventionally used drugs, whose long-term use is associated with severe adverse effects. Then, we expect that this work can bring a new therapeutical option for patients who are refractory to biotechnological drugs and a higher accessibility to better care.

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APPENDIX

1. APPENDIX I

1.1. MULTICOLOR FLOW CYTOMETRY PANEL

TUBO	eF450 [a]	BVMO [b]	BV60F	BV711	FITC [c]	PE	PerCP-Cy 5.5 [d]	PE-Cy7	APC	APC-eF780 [e]
1 29 µL/tube	CD27 5	----	----	----	FVD 4 (1:40)	BAFF-R 5	CD19 5	IgD 2,5	CD32 (FcgRIIB) 5	CD38 2,5
2 26,5 µL/tube	CD27 5	----	----	----	FVD 4 (1:40)	CD21 2,5	CD19 5	IgD 2,5	HLA-DR 5	CD38 2,5
3 29 µL/tube	CD27 5	----	----	----	FVD 4 (1:40)	RANKL 5	CD19 5	IgD 2,5	CD95 5	CD38 2,5
4 27,5 µL/tube	HLA-DR 2,5	----	----	----	FVD 4 (1:40)	CD8 5	CD4 5	CD40-L 5	CD3 5	CD45RO 1
5 17,5 µL/tube + FoxP3 + RORgT	FoxP3 5	----	----	----	FVD 4 (1:40)	RORgT 5	CD4 5	CD25 2,5	CD3 5	CD45RO 1
6 27,5 µL/tube	CCR7 (Pacific Blue) 5	----	----	----	FVD 4 (1:40)	CCR6 5	CD4 5	CXCR3 2,5	CD3 5	CD45RO 1
7 19 µL/tube	HLA-DR 2,5	----	----	----	FVD 4 (1:40)	CD86 2,5	CD14 2,5	----	CD3 5	CD16 2,5
8 29 µL/tube	----	----	----	----	FVD 4 (1:40)	RANK 10	CD14 2,5	CD115 5	CD3 5	CD16 2,5
9 22,5 µL/tube	CD15 1	----	----	----	FVD 4 (1:40)	CXCR2 5	CD11b 2,5	CD66b 5	CD62L 2,5	CD16 2,5
Unstained	----	----	----	----	----	----	----	----	----	----

Tubes		*Populations*
Tubes 1,2,3	→	B cells
Tubes 4,5,6	→	T cells
Tubes 7,8	→	Monocytes
Tubes 9	→	Granulocytes

[a] Pacific Blue, BV421, PB, V450, eF451
[b] PO, V500
[c] Alexa Fluor 488, eF520
[d] PerCP
[e] APC - Cy7

Mix 1 was composed by the following antibodies: CD19 PerCP-Cy5.5, CD27 eFluor450, IgD PE-Cy7, CD38 APC-eFluor 780, BAFF-R PE and FcgRIIB (CD32) APC.

Mix 2 was composed by the following antibodies: CD19 PerCP-Cy5.5, CD27 eFluor450, IgD PE-Cy7, CD38 APC-eFluor 780, CD21 PE and HLA-DR APC.

Mix 3 was composed by the following antibodies: CD19 PerCP-Cy5.5, CD27 eFluor450, IgD PE-Cy7, CD38 APC-eFluor 780, RANKL PE and CD95 APC.

Mix 4 was composed by the following antibodies: CD3 APC, CD4 PerCP-Cy5.5, CD45RO APC-eFluor 780, HLA-DR eFluor 450, CD8 PE and CD40L PE-Cy7.

Mix 5 was composed by the following antibodies: CD3 APC, CD4 PerCP-Cy5.5, CD45RO APC-eFluor 780, Foxp3 eFluor 450, RORgT PE and CD25 PE-Cy7.

Mix 6 was composed by the following antibodies: CD3 APC, CD4 PerCP-Cy5.5, CD45RO APC-eFluor 780, CCR7 Pacific Blue, CCR6 PE and CXCR3 PE-Cy7.

Mix 7 was composed by the following antibodies: CD14 PerCP-Cy5.5, CD3 APC, CD16 APC-Cy7, HLA-DR eFluor 450 and CD86 PE.

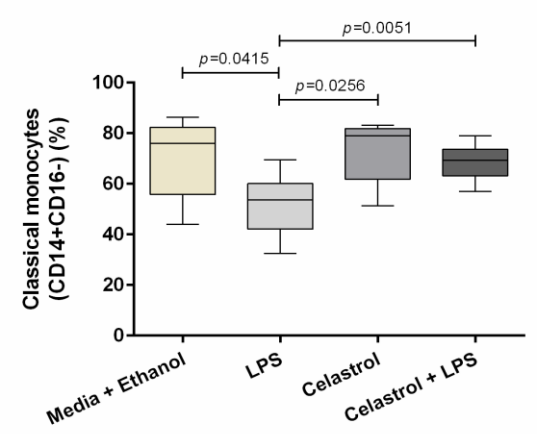
Mix 8 was composed by the following antibodies: CD14 PerCP-Cy5.5, CD3 APC, CD16 APC-Cy7, RANK PE and CD115 PE-Cy7.

Mix 9 was composed by the following antibodies: CD66b PE-Cy7, CD11b PerCP-Cy5.5, CD16 APC-Cy7, CD15 eFluor 450, CXCR2 PE and CD62L APC.

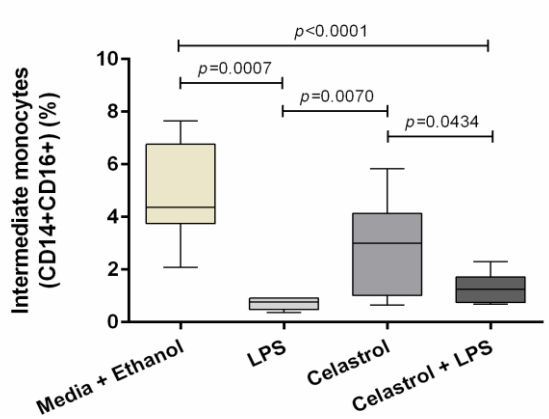
Cell surface or intracellular staining was proceed depending on the type of antibody to be used, being aware that antibodies such as anti- CD3 (1:20), CD4 (1:20), CD8 (1:20), CD11b (1:40), CD14 (1:40), CD15 (1:100), CD16 (1:40), CD19 (1:20), CD21 (1:40), CD25 (1:40), CD27 (1:20), CD32 (FcgRIIB) (1:20), CD38 (1:40), CD40L (1:20), CD45RO (1:40)/ (New : 1:100), CD62-L (L-selectin) (1:40), CD66b (1:20), CD86 (1:40), CD95 (1:20), CD115 (1:20), CXCR2 (CD182) (1:20), CXCR3 (CD183) (1:40), CCR6 (CD196) (1:20), CCR7 (CD197) (1:20), BAFF-R (1:20), HLA-DR (APC - 1:20) (eF450 - 1:40), IgD (1:40), RANK (1:10) and RANKL (1:20) are surface antibodies and anti- Foxp3 (1:20) and RORgT (1:20) are intracellular antibodies.

2. APPENDIX II – Monocytes

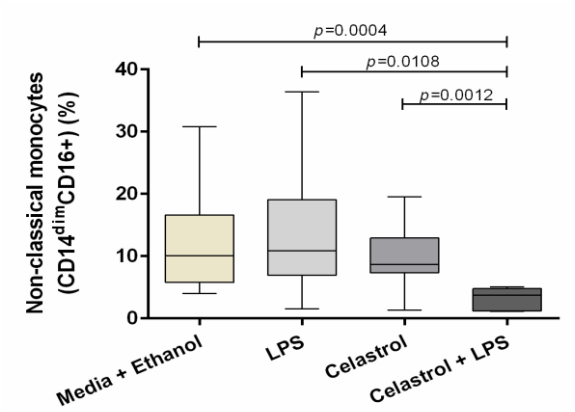
2.1. CELASTROL EFFECT ON MONOCYTES FROM HEALTHY CONTROLS



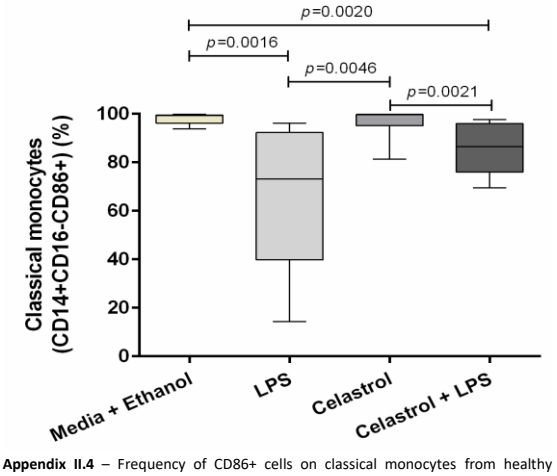
Appendix II.1 – Frequency of classical monocytes from healthy controls is significantly reduced with LPS condition in comparison to Media + Ethanol, and significantly increased with Celastrol and Celastrol + LPS in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



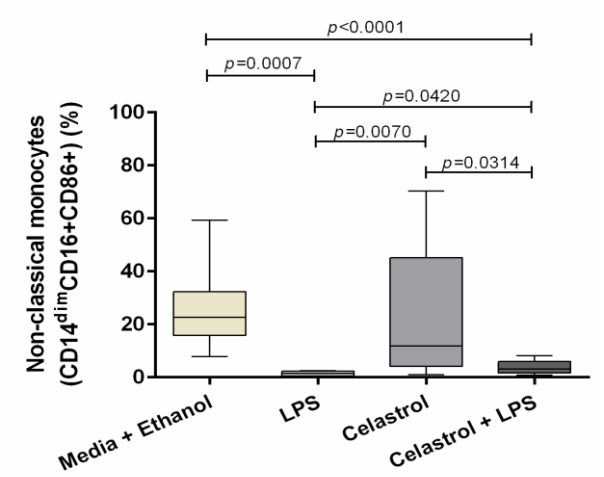
Appendix II.2 – Frequency of intermediate monocytes from healthy controls is significantly reduced with LPS and Celastrol + LPS conditions in comparison to Media + Ethanol, also significantly reduced with Celastrol + LPS in comparison to Celastrol, and significantly increased with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Intermediate monocytes (CD14+CD16+) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



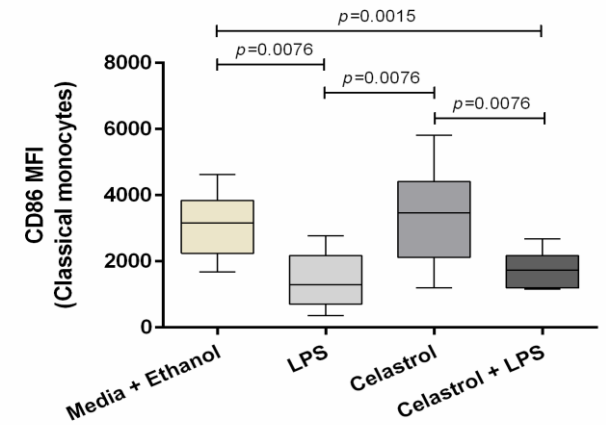
Appendix II.3 – Frequency of non-classical monocytes from healthy controls is significantly reduced with Celastrol + LPS condition in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



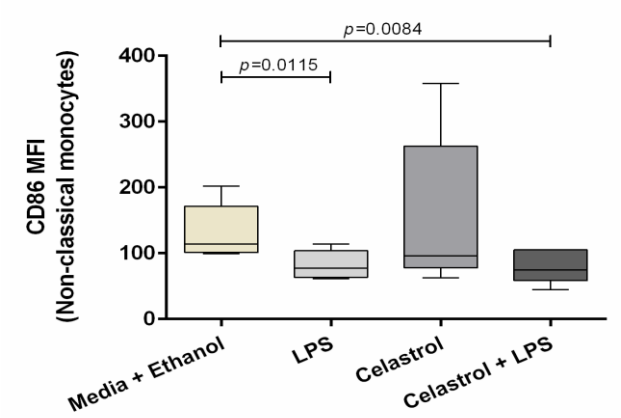
Appendix II.4 – Frequency of CD86+ cells on classical monocytes from healthy controls is significantly reduced with LPS condition in comparison to Media + Ethanol, also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol and Celastrol, and significantly increased with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



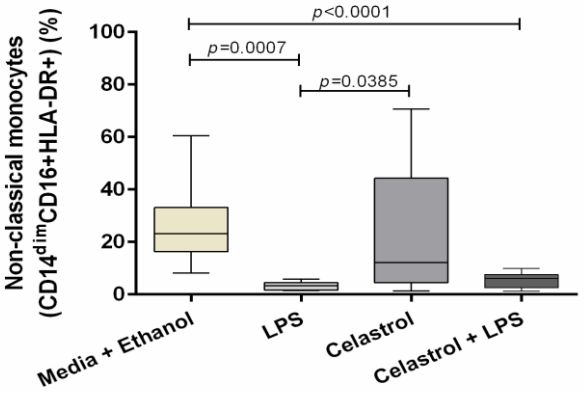
Appendix II.5 – Frequency of CD86+ cells on non-classical monocytes from healthy controls is significantly reduced with LPS condition in comparison to Media + Ethanol, also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol and Celastrol, significantly increased with Celastrol + LPS in comparison to LPS and significantly increased with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



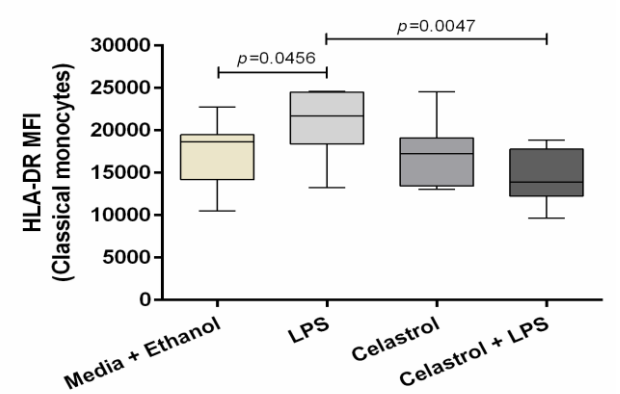
Appendix II.6 – Expression of CD86 (MFI) cell marker on classical monocytes from healthy controls is significantly reduced with LPS in comparison to Media + Ethanol, and also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol and Celastrol, and significantly increased with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests



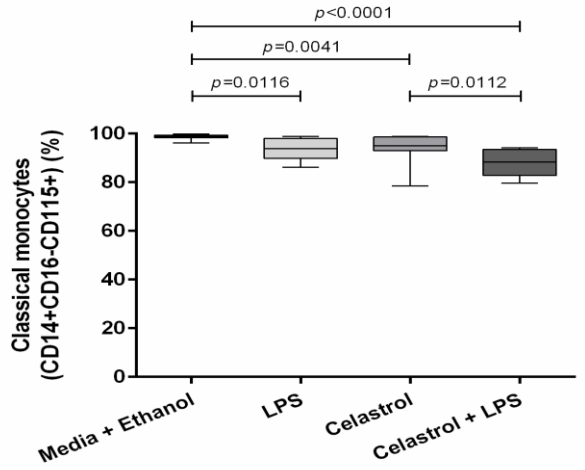
Appendix II.7 – Expression of CD86 (MFI) cell marker on non-classical monocytes from healthy controls is significantly reduced with LPS in comparison to Media + Ethanol and also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



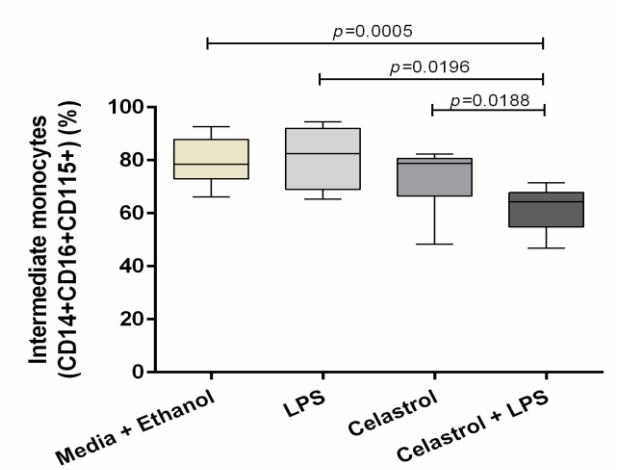
Appendix II.8 – Frequency of HLA-DR+ cells on non-classical monocytes from healthy controls is significantly reduced with LPS condition in comparison to Media + Ethanol, also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol, and significantly increased with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



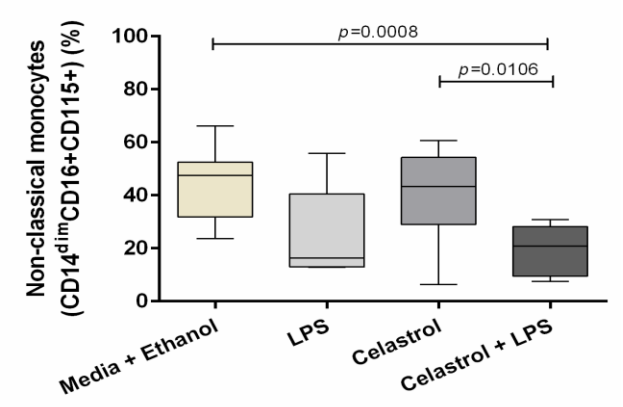
Appendix II.9 – Expression of HLA-DR (MFI) cell marker on classical monocytes from healthy controls is significantly increased with LPS in comparison to Media + Ethanol and significantly reduced with Celastrol + LPS in comparison to LPS. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



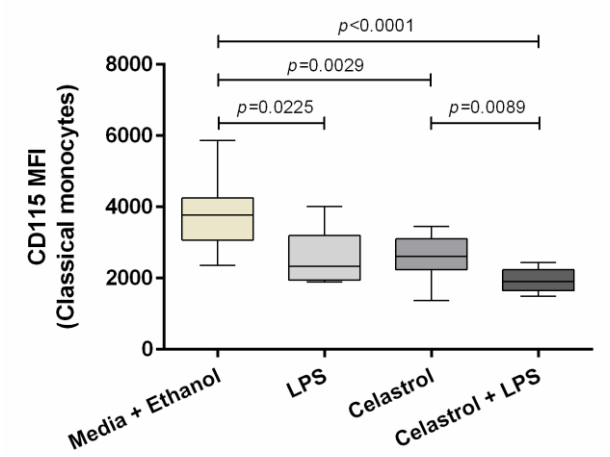
Appendix II.10 – Frequency of CD115+ cells on classical monocytes from healthy controls is significantly reduced with LPS, Celastrol and Celastrol + LPS conditions in comparison to Media + Ethanol and also significantly reduced with Celastrol + LPS in comparison to Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



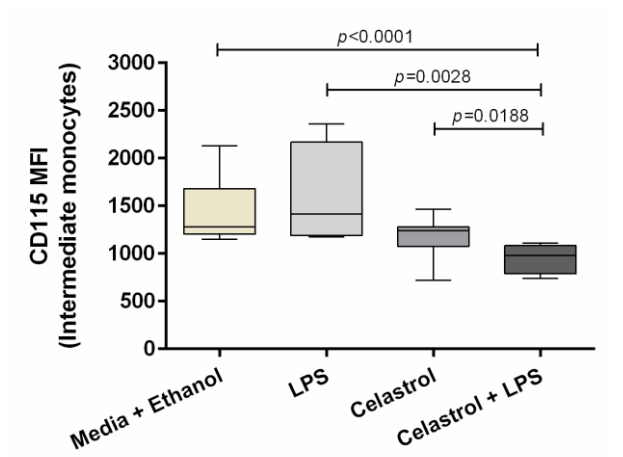
Appendix II.11 – Frequency of CD115+ cells on intermediate monocytes from healthy controls is significantly reduced with Celastrol + LPS condition in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Intermediate monocytes (CD14+CD16+) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



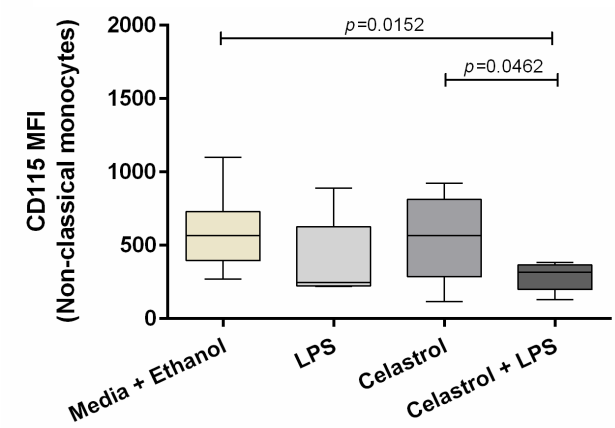
Appendix II.12 – Frequency of CD115+ cells on non-classical monocytes from healthy controls is significantly reduced with Celastrol + LPS condition in comparison to Media + Ethanol and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



Appendix II.13 – Expression of CD115 (MFI) cell marker on classical monocytes from healthy controls is significantly reduced with LPS, Celastrol and Celastrol + LPS in comparison to Media + Ethanol and significantly reduced with Celastrol + LPS in comparison to Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

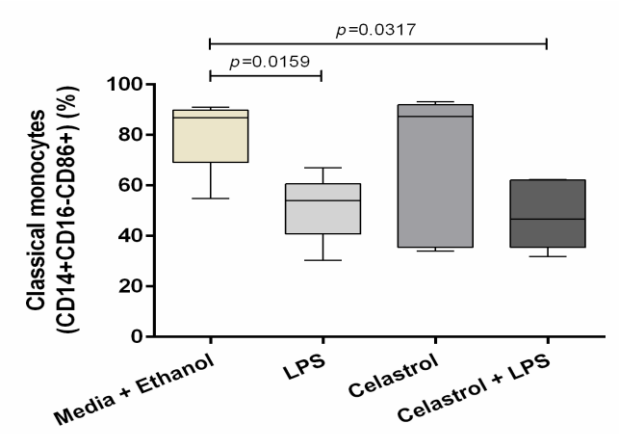


Appendix II.14 – Expression of of CD115 (MFI) cell marker intermediate monocytes from healthy controls is significantly reduced with Celastrol + LPS condition in comparison to Media + Ethanol, LPS and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Intermediate monocytes (CD14+CD16+) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

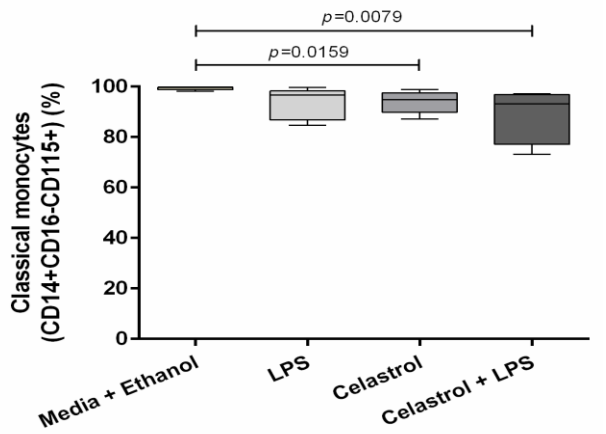


Appendix II.15 – Expression of CD115 (MFI) cell marker on non-classical monocytes from healthy controls is significantly reduced with LPS + Celastrol in comparison to Media + Ethanol and Celastrol. Data from flow cytometry analysis of peripheral blood monocytes from healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

2.2. CELASTROL EFFECT ON MONOCYTES FROM CHRONIC RA PATIENTS

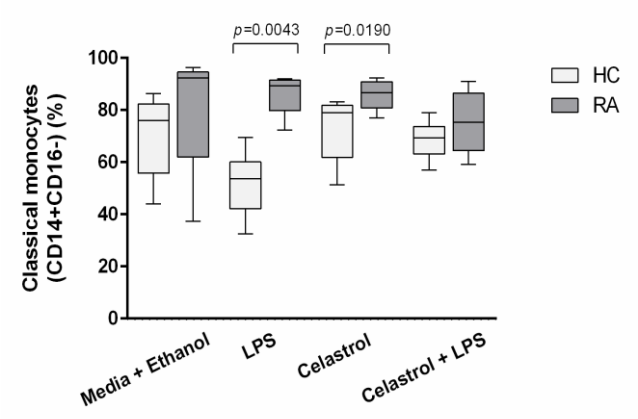


Appendix II.16 – Frequency of CD86+ cells on classical monocytes from chronic RA patients is significantly reduced with LPS condition in comparison to Media + Ethanol, also significantly reduced with Celastrol + LPS in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

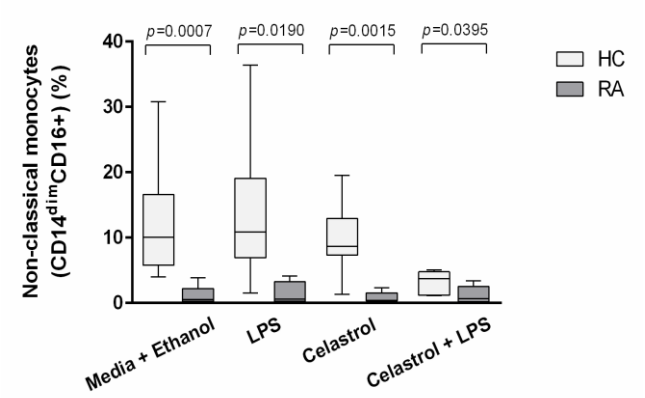


Appendix II.17 – Frequency of CD115+ cells on classical monocytes from chronic RA patients is significantly reduced with Celastrol and Celastrol + LPS conditions in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

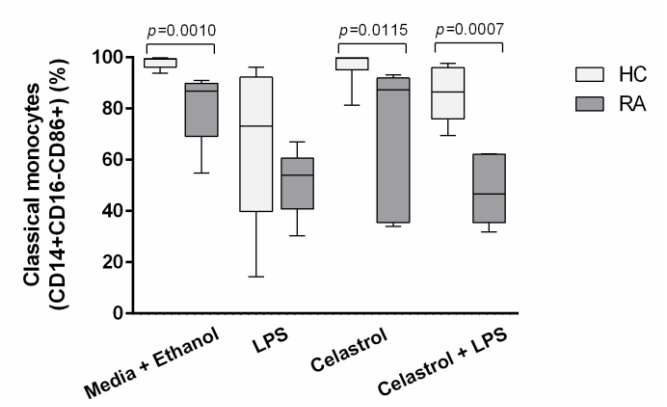
2.3. CELASTROL EFFECT ON MONOCYTES FROM CHRONIC RA PATIENTS *vs* HEALTHY CONTROLS



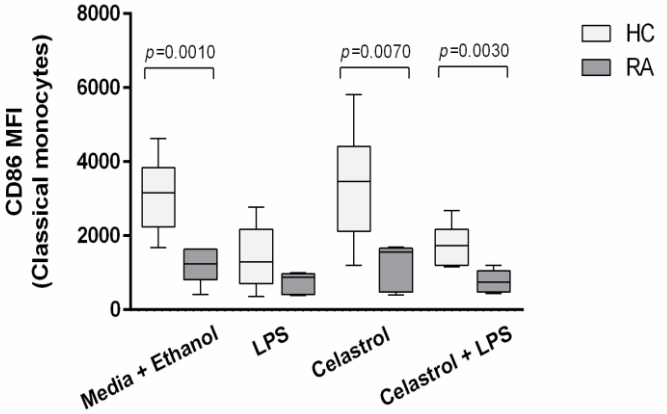
Appendix II.18 – Frequency of classical monocytes in chronic RA patients tested with LPS and Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



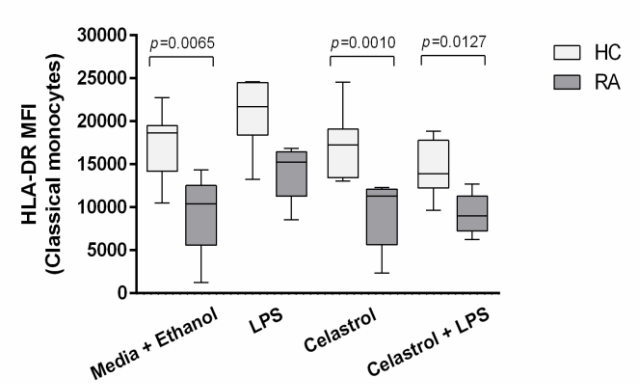
Appendix II.19 – Frequency of non-classical monocytes in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



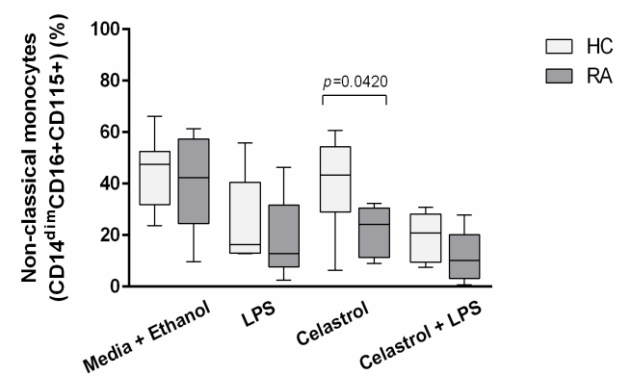
Appendix II.20 – Frequency of CD86+ cells on classical monocytes in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



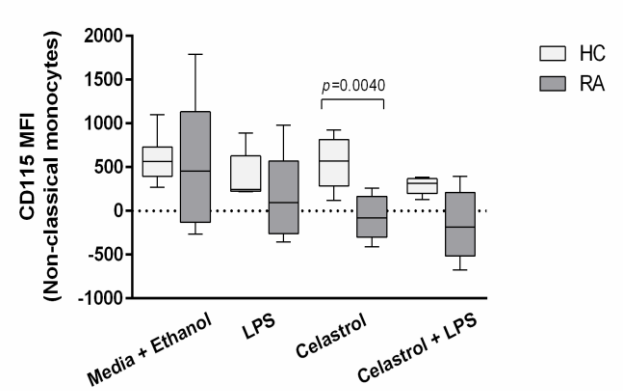
Appendix II.21 – Expression of CD86 (MFI) cell marker on classical monocytes in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



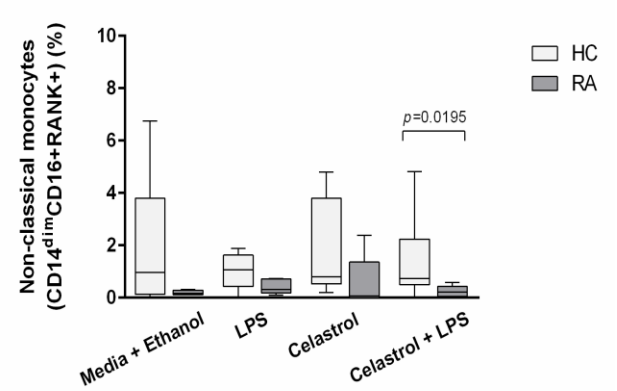
Appendix II.22 – Expression of HLA-DR (MFI) cell marker on classical monocytes in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Classical monocytes (CD14+CD16-) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



Appendix II.23 – Frequency of CD115+ cells on non-classical monocytes in chronic RA patients tested with Celastrol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



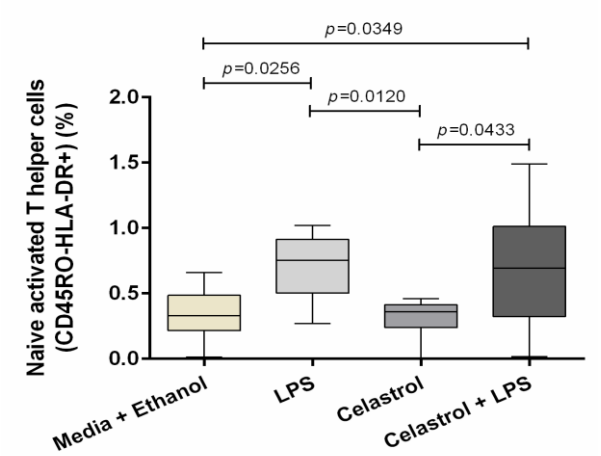
Appendix II.24 – Expression of CD115 (MFI) cell marker on non-classical monocytes in chronic RA patients tested with Celastrol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



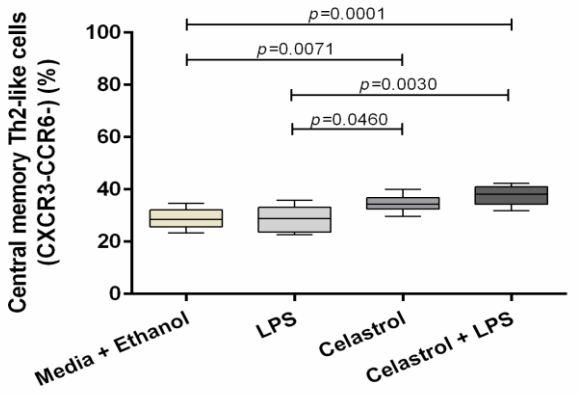
Appendix II.25 – Frequency of RANK+ cells on non-classical monocytes in chronic RA patients tested with Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood monocytes from chronic RA patients and healthy controls tested with several conditions. Non-classical monocytes ($CD14^{dim}CD16+$) were gated in CD14+ cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

3. APPENDIX III – T cells

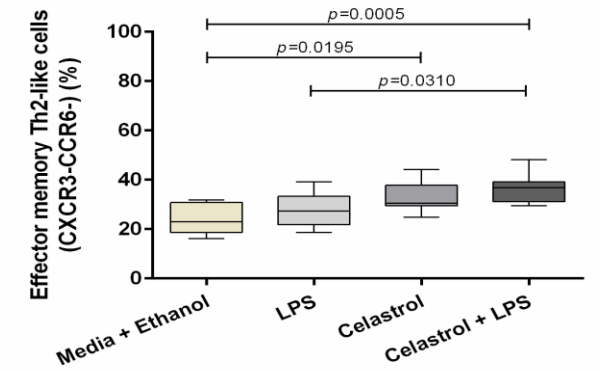
3.1. CELASTROL EFFECT ON T CELLS FROM HEALTHY CONTROLS



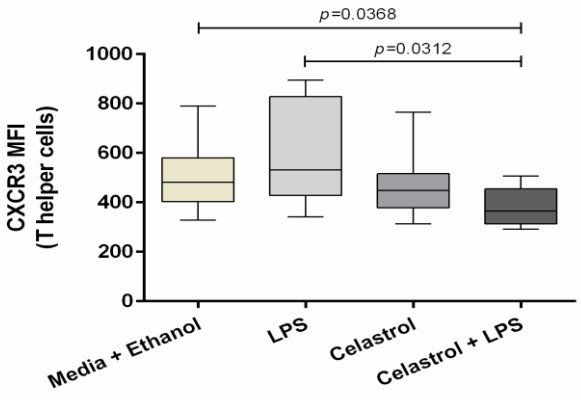
Appendix III.1 – Frequency of naïve activated T helper cells from healthy controls is significantly reduced with Celastrol condition in comparison to LPS and significantly increased with Celastrol + LPS in comparison to Media + Ethanol and Celastrol. Data from flow cytometry analysis of peripheral blood T cells from healthy controls tested with several conditions. Naïve activated T helper cells (CD45RO-HLA-DR+) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



Appendix III.2 – Frequency of central memory Th2-like cells from healthy controls is significantly increased with Celastrol condition in comparison to Media + Ethanol and LPS, and also significantly increased with Celastrol + LPS in comparison to Media + Ethanol and LPS. Data from flow cytometry analysis of peripheral blood T cells from healthy controls tested with several conditions. Central memory Th2-like cells (CD45RO+CCR7+CXCR3-CCR6-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

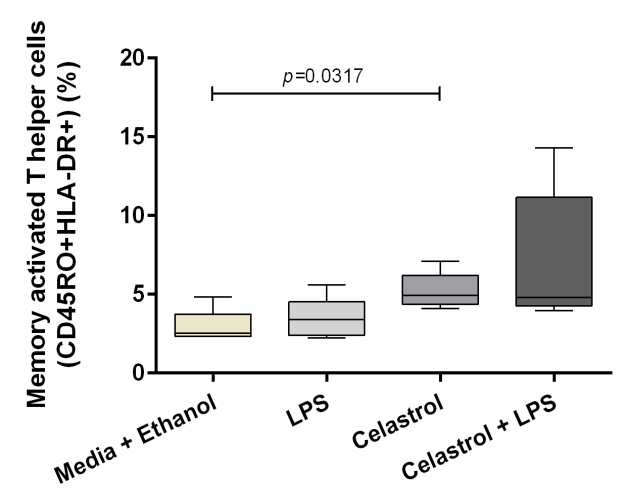


Appendix III.3 – Frequency of effector memory Th2-like cells from healthy controls is significantly increased with Celastrol condition in comparison to Media + Ethanol and also significantly increased with Celastrol + LPS in comparison to Media + Ethanol and LPS. Data from flow cytometry analysis of peripheral blood T cells from healthy controls tested with several conditions. Effector memory Th2-like cells (CD45RO+CCR7-CXCR3-CCR6-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



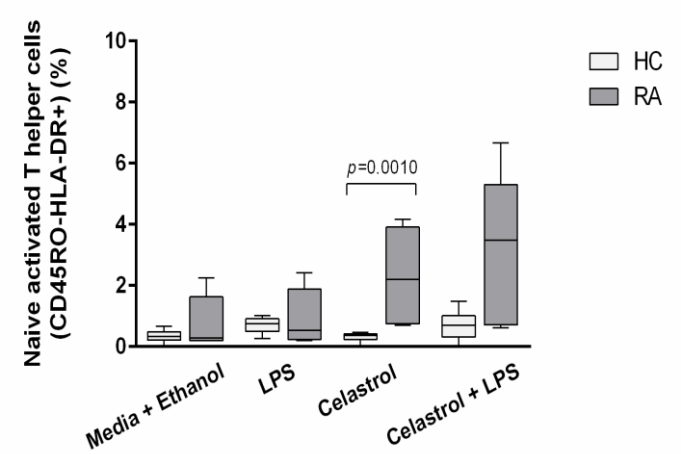
Appendix III.4 – Expression of CXCR3 (MFI) cell marker on T helper cells from healthy controls is significantly reduced with Celastrol + LPS in comparison to Media + Ethanol and LPS. Data from flow cytometry analysis of peripheral blood T cells from healthy controls tested with several conditions. T helper cells (CD3+CD4+) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

3.2. CELASTROL EFFECT ON T CELLS FROM CHRONIC RA PATIENTS

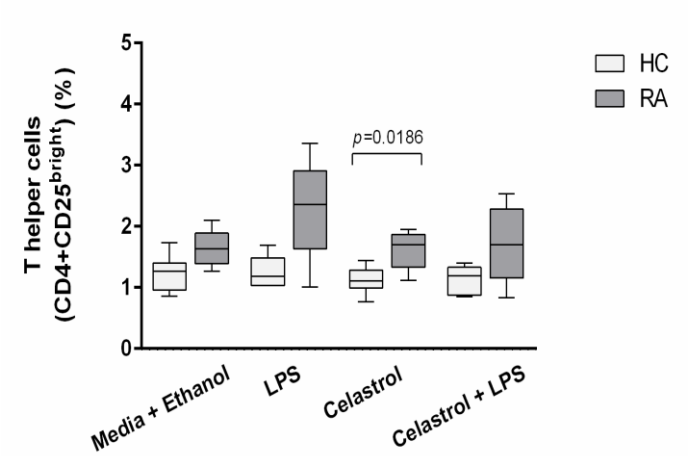


Appendix III.5 – Frequency of memory activated T helper cells from chronic RA patients is significantly increased with Celastrol + LPS condition in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients tested with several conditions. Memory activated T helper cells (CD45RO+HLA-DR+) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

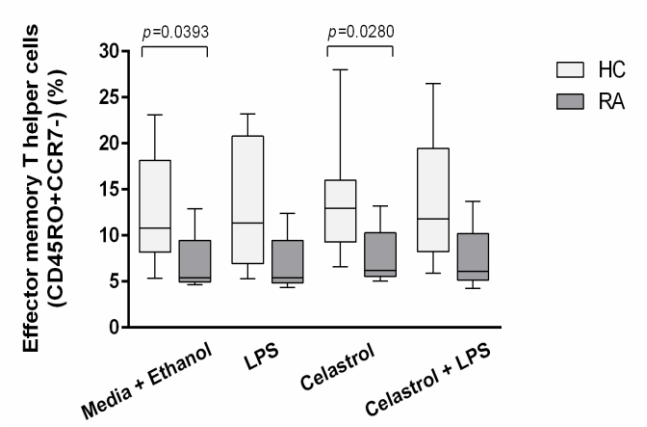
3.3. CELASTROL EFFECT ON T CELLS FROM CHRONIC RA PATIENTS *vs* HEALTHY CONTROLS



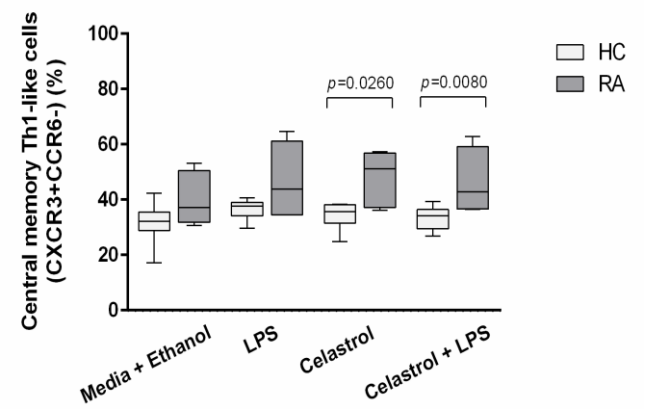
Appendix III.6 – Frequency of naïve activated T helper cells in chronic RA patients tested with Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Naïve activated T helper cells (CD45RO-HLA-DR+) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



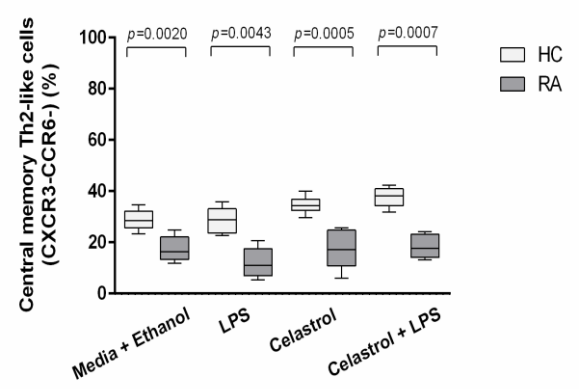
Appendix III.7 – Frequency of $CD25^{bright}$ regulatory T cells in chronic RA patients tested with Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. $CD25^{bright}$ regulatory T cells ($CD4+CD25^{bright}$) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



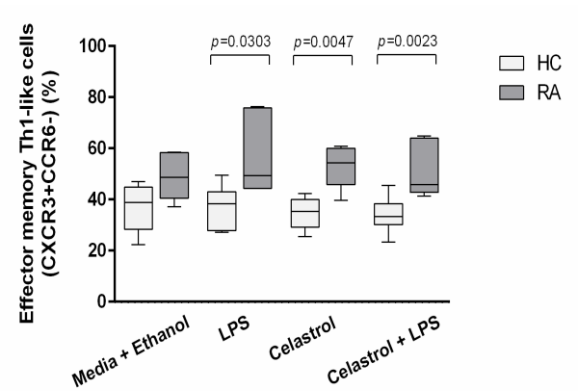
Appendix III.8 – Frequency of effector memory T helper cells in chronic RA patients tested with Media + Ethanol and Celastrol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Effector memory T helper cells (CD45RO+CCR7-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



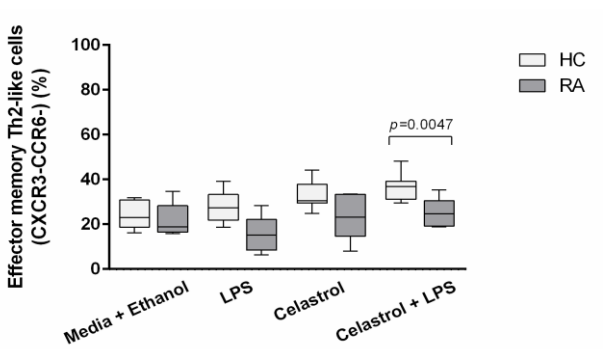
Appendix III.9 – Frequency of central memory Th1-like cells in chronic RA patients tested with Celastol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Central memory Th1-like cells (CD45RO+CCR7+CXCR3+CCR6-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



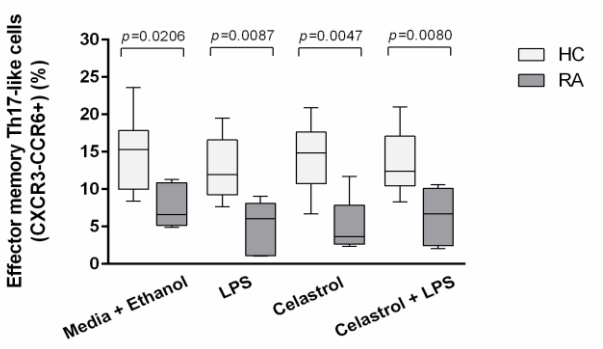
Appendix III.10 – Frequency of central memory Th2-like cells in chronic RA patients tested with Media + Ethanol, LPS, Celastol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Central memory Th2-like cells (CD45RO+CCR7+CXCR3-CCR6-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



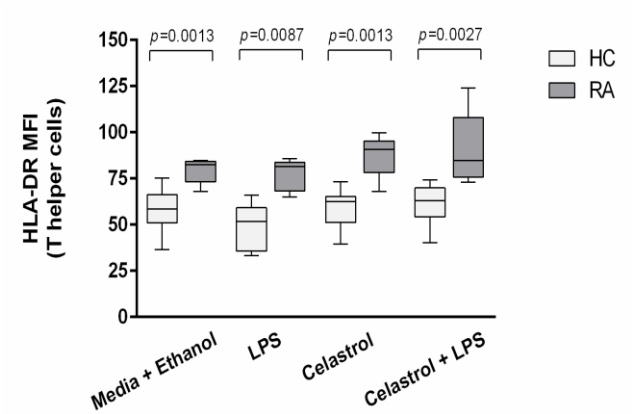
Appendix III.11 – Frequency of effector memory Th1-like cells in chronic RA patients tested with LPS, Celastol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Effector memory Th1-like cells (CD45RO+CCR7-CXCR3+CCR6-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



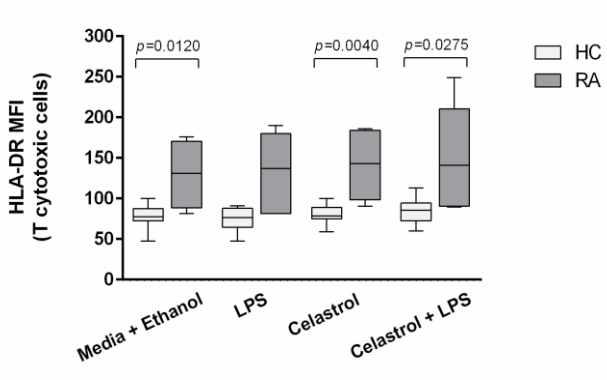
Appendix III.12 – Frequency of effector memory Th2-like cells in chronic RA patients tested with Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Effector memory Th2-like cells (CD45RO+CCR7-CXCR3-CCR6-) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



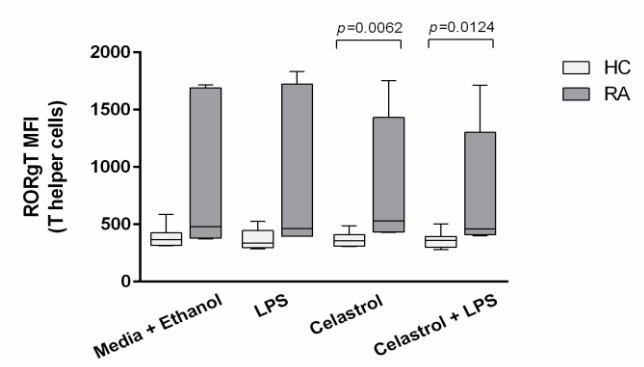
Appendix III.13 – Frequency of effector memory Th17-like cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Effector memory Th17-like cells (CD45RO+CCR7-CXCR3-CCR6+) were gated in CD3+CD4+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



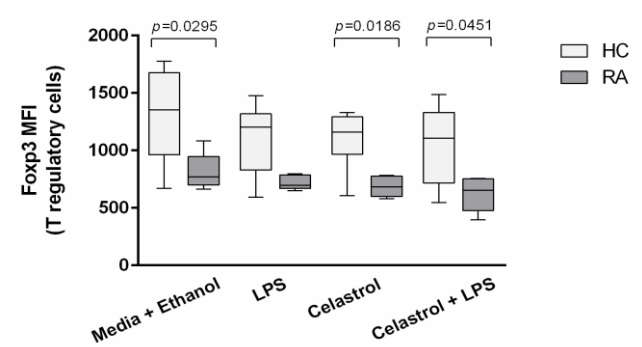
Appendix III.14 – Expression of HLA-DR (MFI) cell marker on T helper cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. T helper cells (CD3+CD4+) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



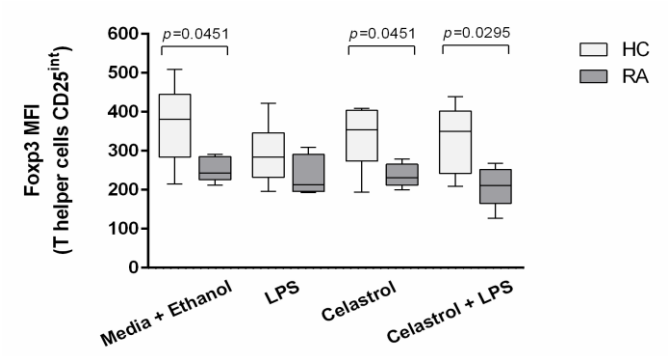
Appendix III.15 – Expression of HLA-DR (MFI) cell marker on T cytotoxic cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. T cytotoxic cells (CD3+CD8+) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



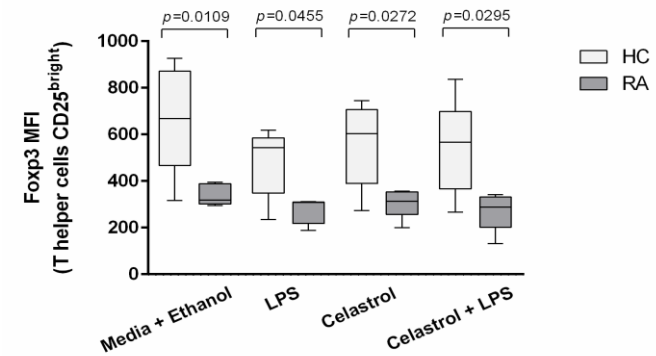
Appendix III.16 – Expression of RORgT (MFI) cell marker on T helper cells in chronic RA patients tested with Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. T helper cells (CD3+CD4+) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



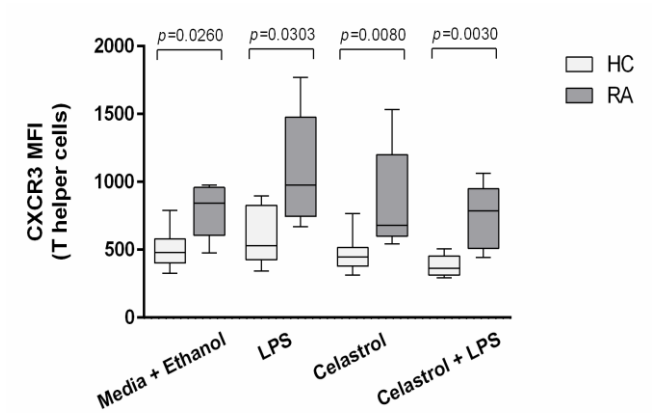
Appendix III.17 – Expression of Foxp3 (MFI) cell marker on regulatory T cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. Regulatory T cells (CD25+Foxp3+ subset) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-



Appendix III.18 – Expression of Foxp3 (MFI) cell marker on $CD25^{int}$ regulatory T cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. $CD25^{int}$ regulatory T cells ($CD4+CD25^{int}$) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey



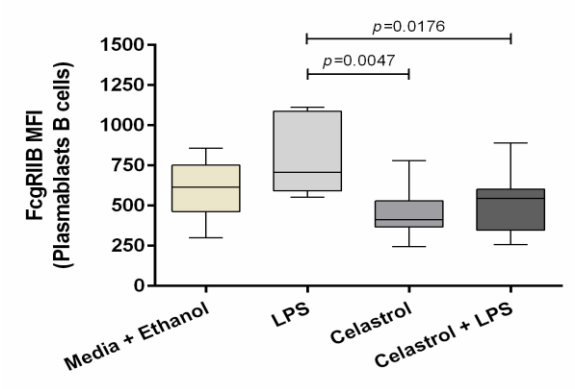
Appendix III.19 – Expression of Foxp3 (MFI) cell marker on $CD25^{bright}$ regulatory T cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. $CD25^{bright}$ regulatory T cells ($CD4+CD25^{bright}$) were gated in CD3+ T cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



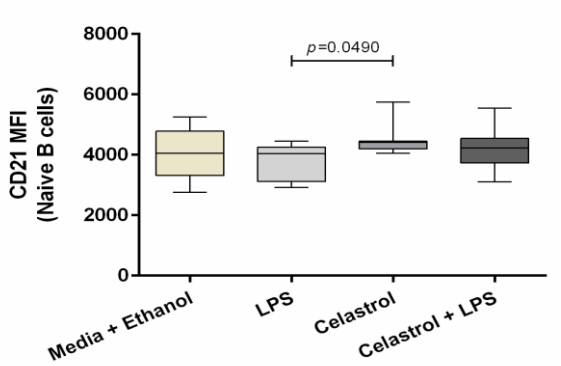
Appendix III.20 – Expression of CXCR3 (MFI) cell marker on T helper cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood T cells from chronic RA patients and healthy controls tested with several conditions. T helper cells (CD3+CD4+) were gated in CD3+ T cells Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

4. APPENDIX IV – B cells

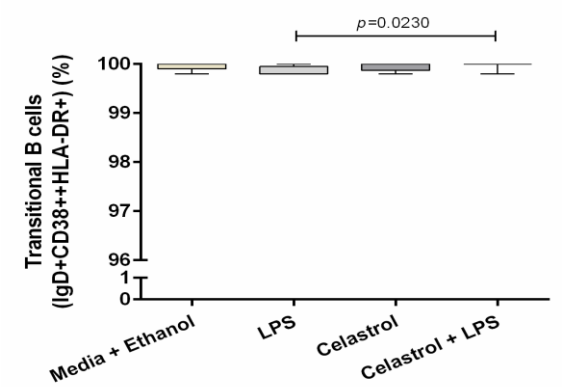
4.1. CELASTROL EFFECT ON B CELLS FROM HEALTHY CONTROLS



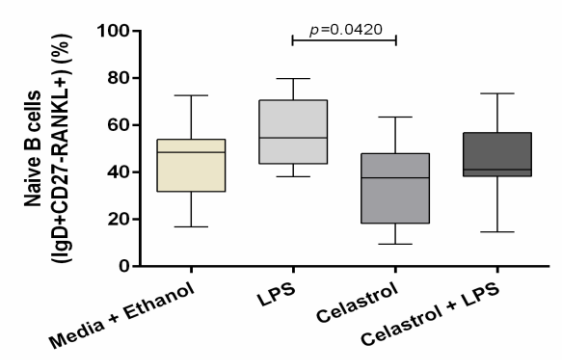
Appendix IV.1 – Expression of FcgRIIB (MFI) cell marker on plasmablasts from healthy controls is significantly reduced with Celastrol and Celastrol + LPS conditions in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Plasmablasts (IgD-CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



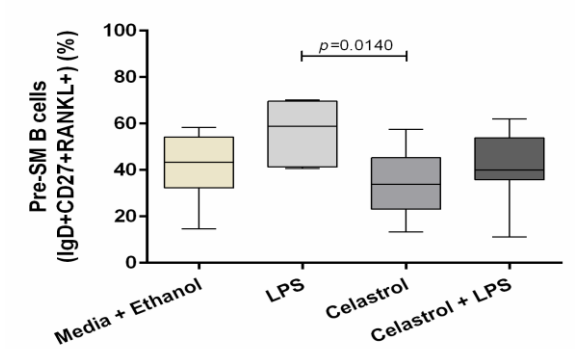
Appendix IV.2 – Expression of CD21 (MFI) cell marker on naïve B cells from healthy controls is significantly increased with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests



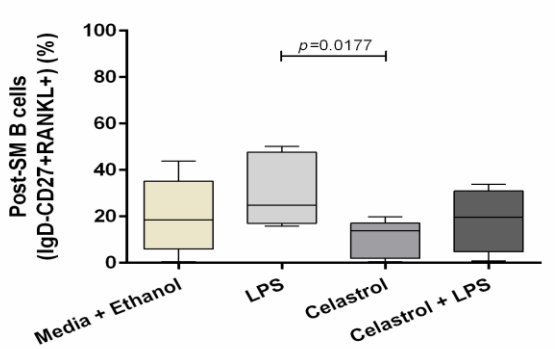
Appendix IV.3 – Frequency of HLA-DR+ cells on transitional B cells from healthy controls is significantly increased with Celastrol + LPS condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Transitional B cells (IgD+CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



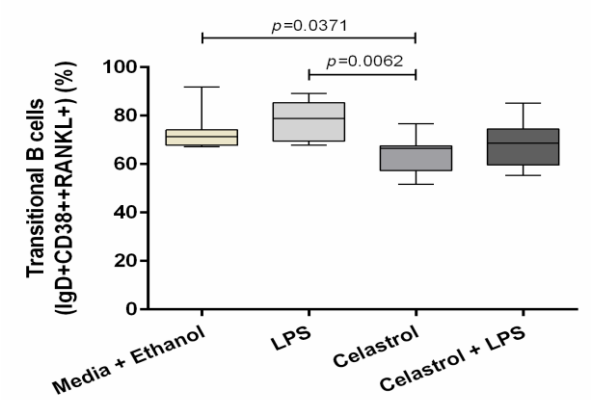
Appendix IV.4 – Frequency of RANKL+ cells on naïve B cells from healthy controls is significantly increased with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



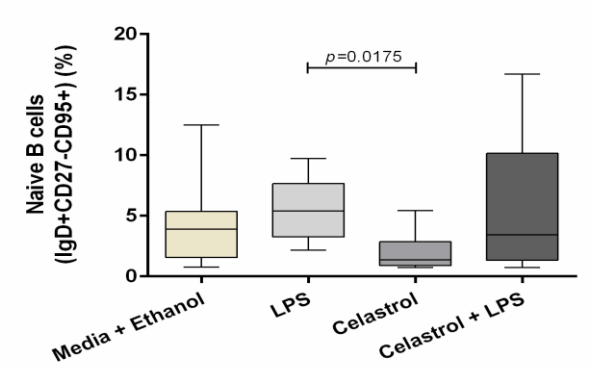
Appendix IV.5 – Frequency of RANKL+ cells on pre-switch memory B cells from healthy controls is significantly reduced with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Pre-switch memory B cells (IgD+CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



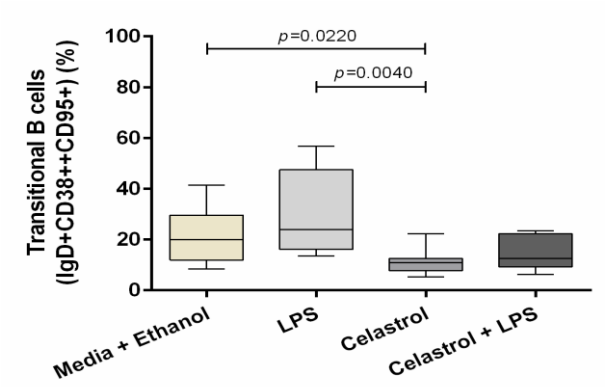
Appendix IV.6– Frequency of RANKL+ cells on post-switch memory B cells from healthy controls is significantly reduced with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Post--switch memory B cells (IgD-CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



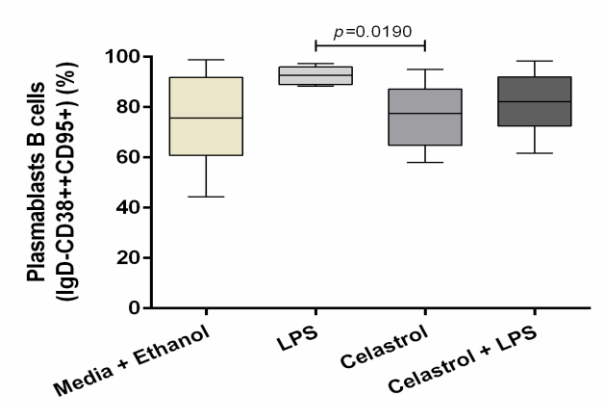
Appendix IV.7– Frequency of RANKL+ cells on transitional B cells from healthy controls is significantly reduced with Celastrol condition in comparison to Media + Ethanol and LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Transitional B cells (IgD+CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



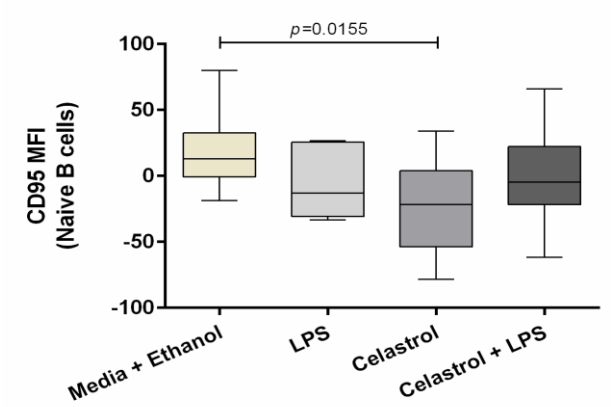
Appendix IV.8 – Frequency of CD95+ cells on naïve B cells from healthy controls is significantly reduced with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



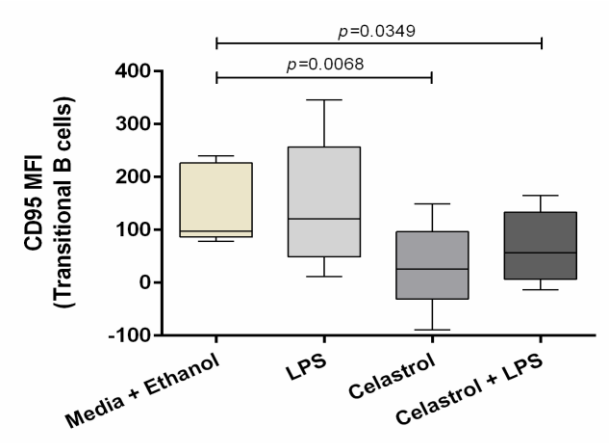
Appendix IV.9 – Frequency of CD95+ cells on transitional B cells from healthy controls is significantly reduced with Celastrol condition in comparison to Media + Ethanol and LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Transitional B cells (IgD+CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



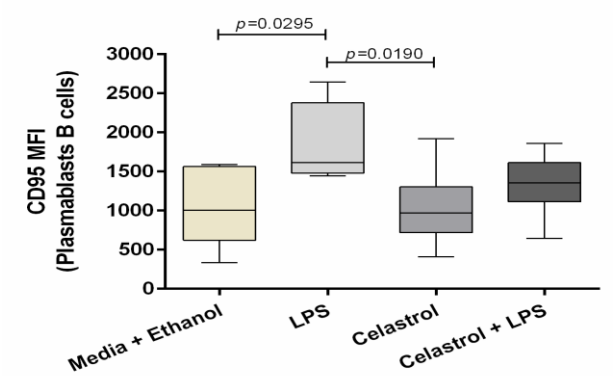
Appendix IV.10 – Frequency of CD95+ cells on plasmablasts from healthy controls is significantly reduced with Celastrol condition in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Plasmablasts (IgD-CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



Appendix IV.11 – Expression of CD95 (MFI) cell marker on naïve B cells from healthy controls is significantly reduced with Celastrol condition in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

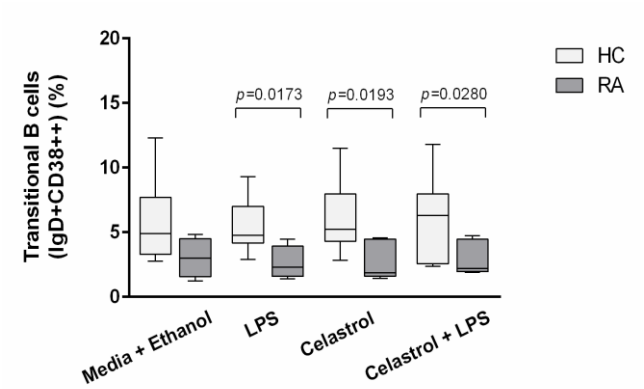


Appendix IV.12 – Expression of CD95 (MFI) cell marker on transitional B cells from healthy controls is significantly reduced with Celastrol and Celastrol + LPS conditions in comparison to Media + Ethanol. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Transitional B cells (IgD+CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

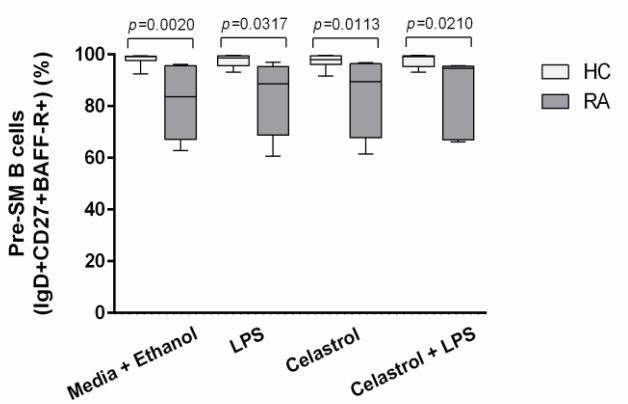


Appendix IV.13 – Expression of CD95 (MFI) cell marker on plasmablasts from healthy controls is significantly increased with LPS in comparison to Media + Ethanol and is significantly reduced with Celastrol in comparison to LPS. Data from flow cytometry analysis of peripheral blood B cells from healthy controls tested with several conditions. Plasmablasts (IgD-CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

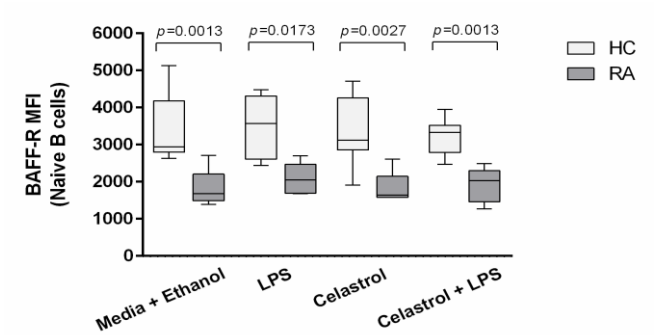
4.2. CELASTROL EFFECT ON B CELLS FROM CHRONIC RA PATIENTS *vs* HEALTHY CONTROLS



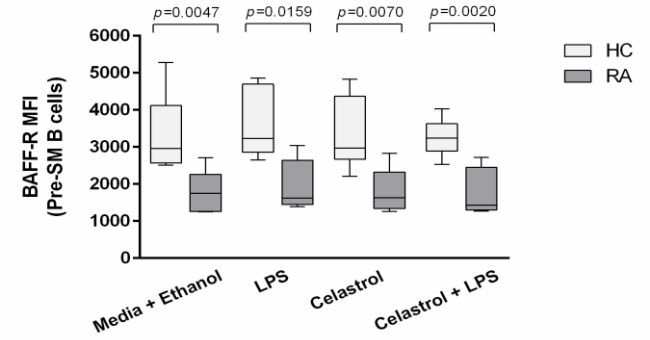
Appendix IV.14 – Frequency of transitional B cells in chronic RA patients tested with LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Transitional B cells (IgD+CD38++) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



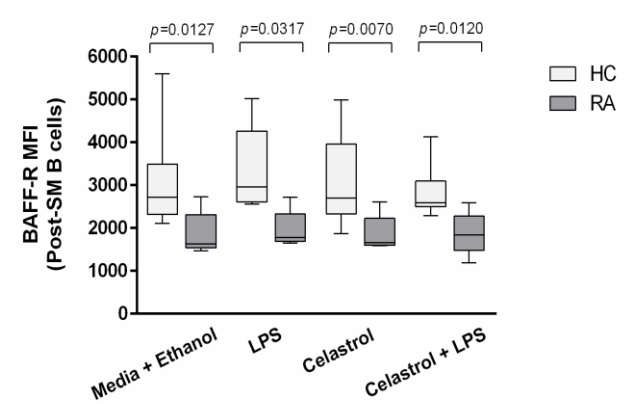
Appendix IV.15 – Frequency of BAFF-R+ cells on pre-switch memory B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Pre-switch memory B cells (IgD+CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



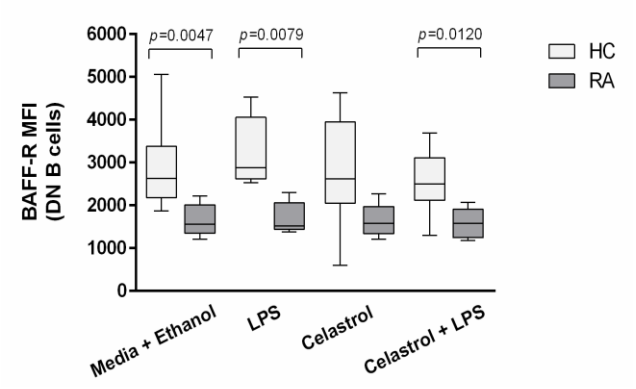
Appendix IV.16 – Expression of BAFF-R (MFI) cell marker on naïve B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



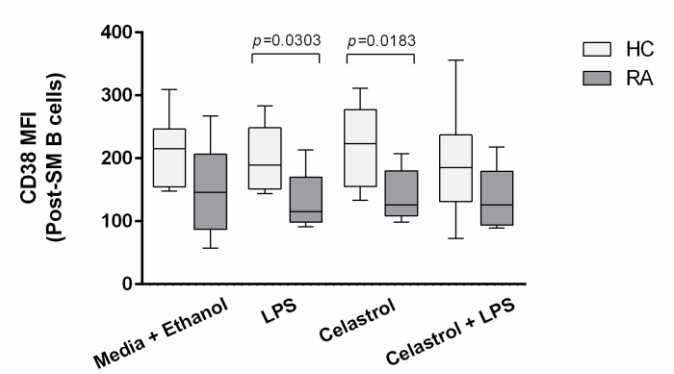
Appendix IV.17 – Expression of BAFF-R (MFI) cell marker on pre-switch memory B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Pre-switch memory B cells (IgD+CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



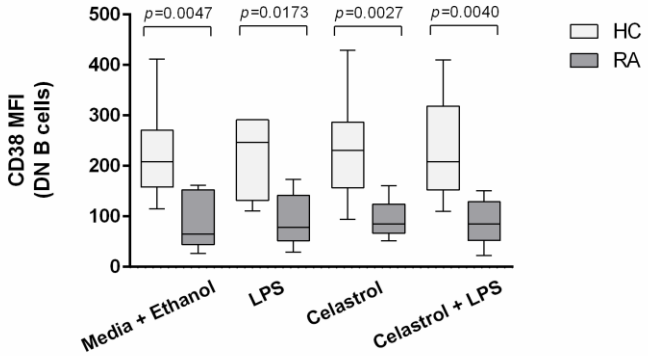
Appendix IV.18 – Expression of BAFF-R (MFI) cell marker on post-switch memory B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Post-switch memory B cells (IgD-CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



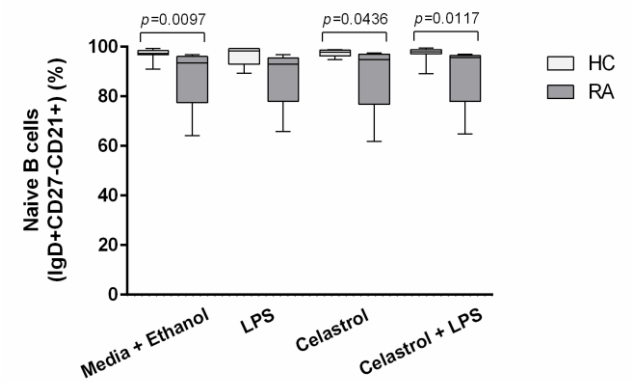
Appendix IV.19 – Expression of BAFF-R (MFI) cell marker on double-negative B cells in chronic RA patients tested with Media + Ethanol, LPS and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Double-negative B cells (IgD-CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



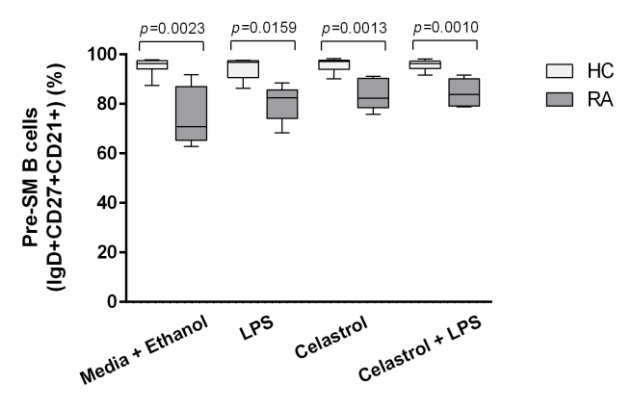
Appendix IV.20 – Expression of CD38 (MFI) cell marker on post-switch memory B cells in chronic RA patients tested with LPS and Celastrol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Post-switch memory B cells (IgD-CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



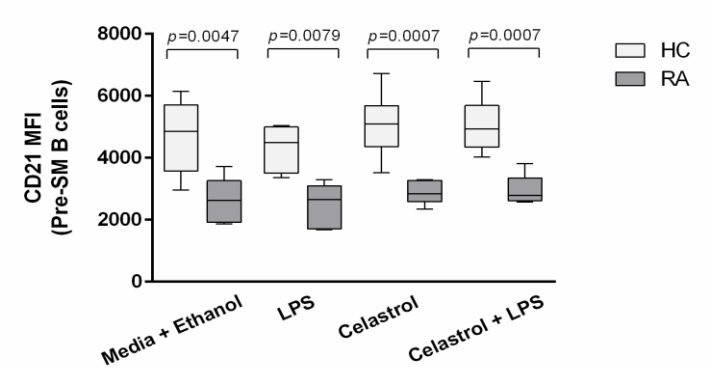
Appendix IV.21 – Expression of CD38 (MFI) cell marker on double-negative B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Double-negative B cells (IgD-CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



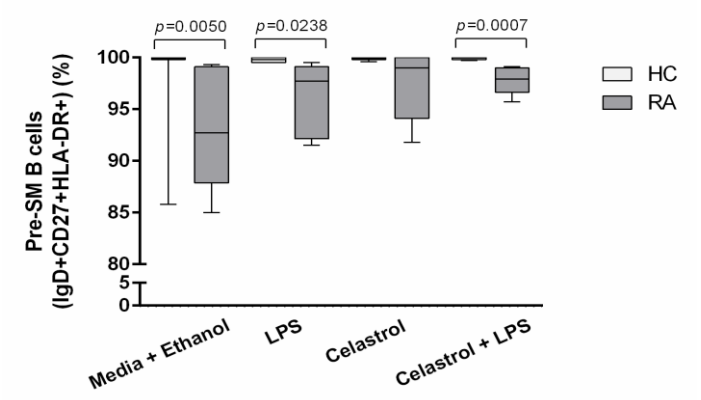
Appendix IV.22 – Frequency of CD21+ cells on naïve B cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



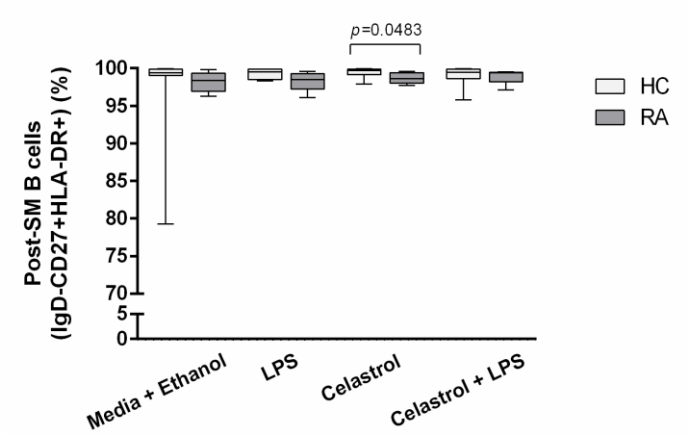
Appendix IV.23 – Frequency of CD21+ cells on pre-switch memory B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Pre-switch memory B cells (IgD+CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



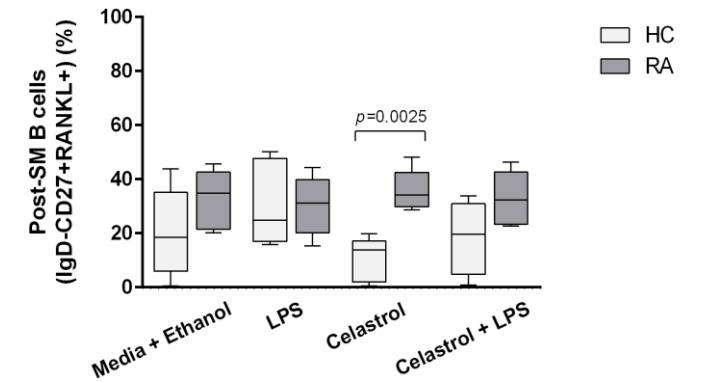
Appendix IV.24 – Expression of CD21 (MFI) cell marker on pre-switch memory B cells in chronic RA patients tested with Media + Ethanol, LPS, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Pre-switch memory B cells (IgD+CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



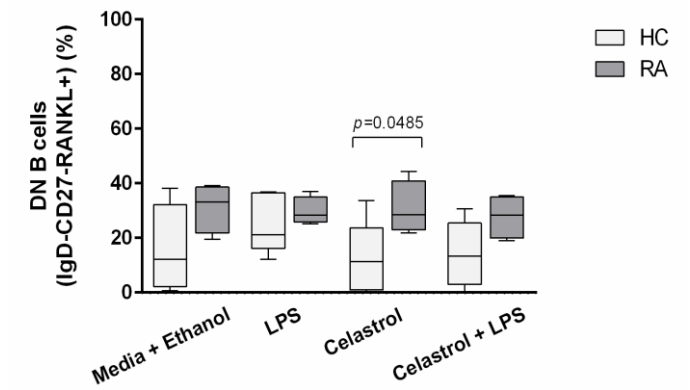
Appendix IV.25 – Frequency of HLA-DR+ cells on pre-switch memory B cells in chronic RA patients tested with Media + Ethanol, LPS and Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Pre-switch memory B cells (IgD+CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



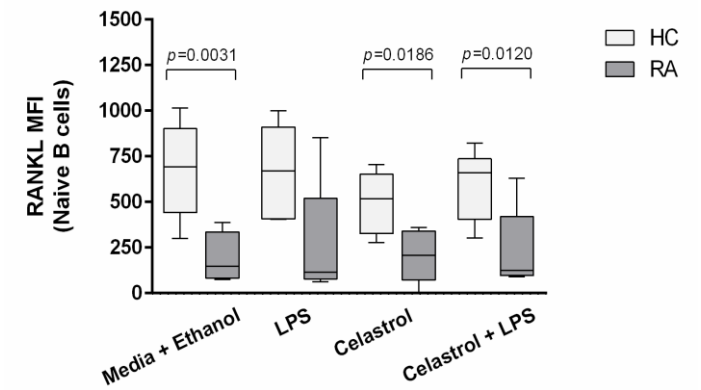
Appendix IV.26 – Frequency of HLA-DR+ cells on post-switch memory B cells in chronic RA patients tested with Celastrol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Post-switch memory B cells (IgD-CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



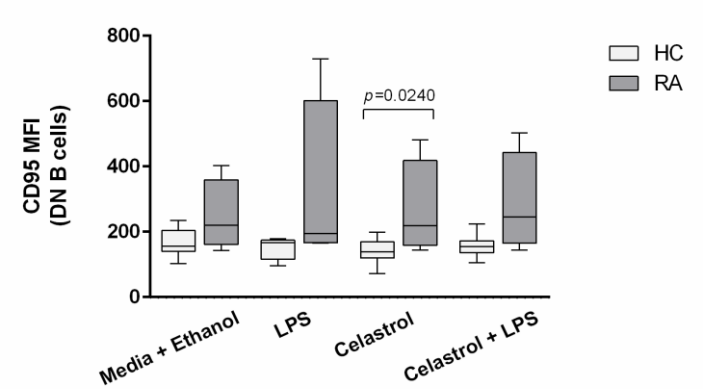
Appendix IV.27 – Frequency of RANKL+ cells on post-switch memory B cells in chronic RA patients tested with Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Post-switch memory B cells (IgD-CD27+) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



Appendix IV.28 – Frequency of RANKL+ cells on double-negative B cells in chronic RA patients tested with Celastrol is significantly increased in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Double-negative B cells (IgD-CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



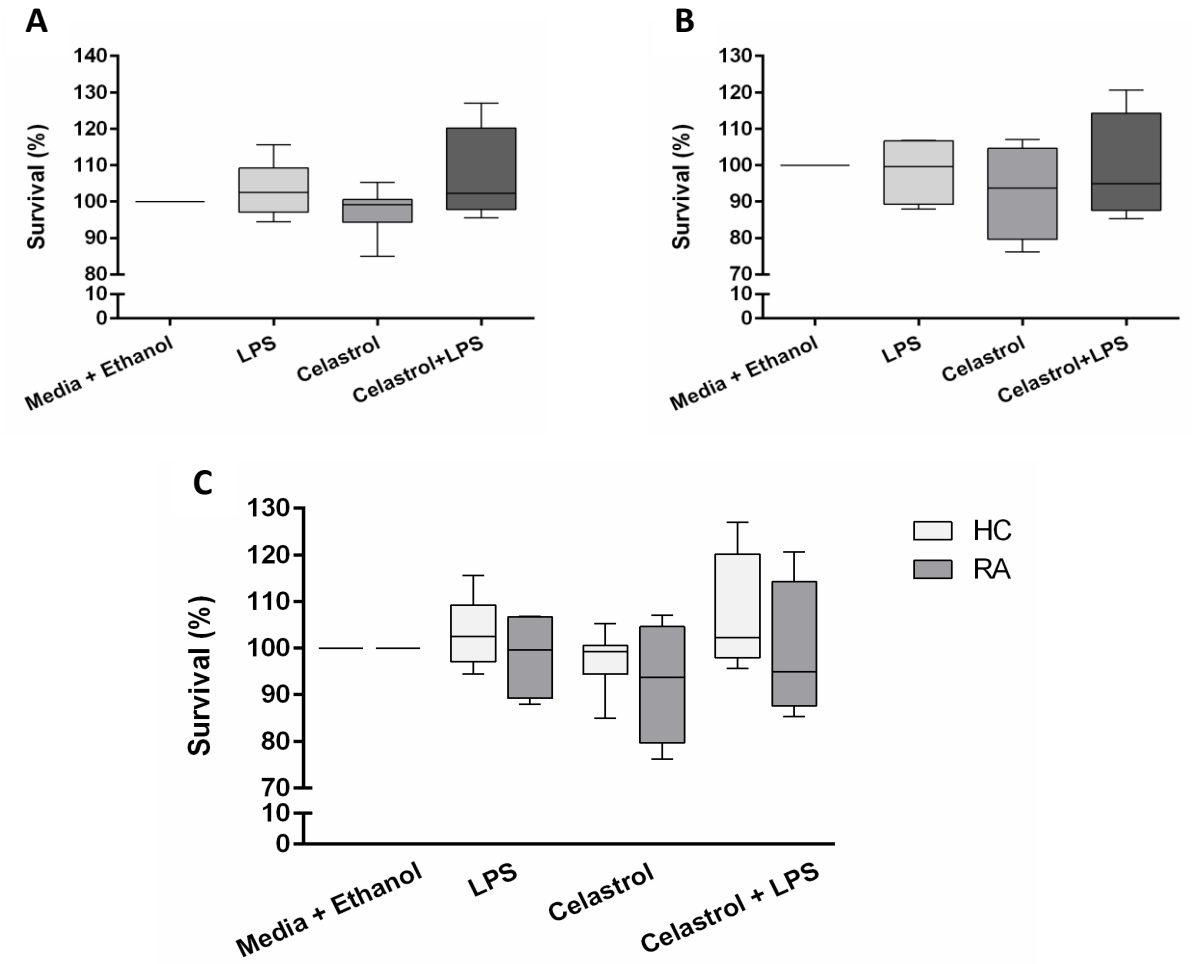
Appendix IV.29 – Expression of RANKL (MFI) cell marker on naïve B cells in chronic RA patients tested with Media + Ethanol, Celastrol and Celastrol + LPS is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Naïve B cells (IgD+CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.



Appendix IV.30 – Expression of CD95 (MFI) cell marker on double-negative B cells in chronic RA patients tested with Media + Ethanol is significantly reduced in comparison to healthy controls. Data from flow cytometry analysis of peripheral blood B cells from chronic RA patients and healthy controls tested with several conditions. Double-negative B cells (IgD-CD27-) were gated in CD19+ B cells. Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.

5. APPENDIX V

5.1. LEUKOCYTES VIABILITY AFTER *IN VITRO* TEST OF CELASTROL – AlamarBlue® assay



Appendix V.1 – **Leucokytes viability after *in vitro* test of celastrol**. **Cell viability analysis by alarmarBlue® assay. (A)** Healthy controls, *n*=10. **(B)** Chronic RA patients, *n*=5. **(C)** Chronic RA patients *vs* Healthy controls. There were no statistically significant differences ($p<0.05$) in cell viability between the tested experimental conditions, either in healthy controls, chronic RA patients or healthy controls *vs* chronic RA patients. Thus, these data indicate that the optimized experimental se-tup does not affect the results of the experiment. Both the addition of the LPS stimulus, celastrol and media maintain cell viability in the same range (above 80%, 90% viability). **Data from the alarmarBlue® cell viability assay.** Data are expressed as median with interquartile range. Differences were considered statistically significant for *p*-values<0.05 according to the Mann-Whithey tests.